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# EXPERIMENTAL GLOMERULONEPHRITIS*

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There are four anatomic types of renal disease in man that are commonly called nephritis. These differ in etiology as well as in clinical and anatomic features, and it is therefore important to distinguish them sharply in an experimental study.

1. Exudative interstitial nephritis (pyelonephritis, abscess). This is an ordinary exudative inflammation of the kidney in which bacteria are readily demonstrable. Acute interstitial nephritis is a special type of exudative inflammation most frequently observed following scarlet fever.

The exudate, composed of polymorphonuclear and mononuclear leucocytes, compresses and destroys portions of the tubules and may in this way produce areas of atrophy in chronic cases. In man contracted kidneys are rarely if ever produced by this disease; but in the laboratory animals this is the only known form of contracted kidney.

The spontaneous nephritis [1] of the rabbit is of the exudative interstitial type. It is readily produced by intravenous injections of streptococci and seems to develop frequently when the kidneys are injured by toxic chemical substances such as uranium nitrate. This is the only form of contracted kidney that has been obtained by experimental procedures. It is a chronic nephritis but it is quite different histologically from the human contracted kidney.

2. Nephrosis. This is a degenerative change, affecting chiefly the tubules, but to some extent the glomeruli also, and varying in intensity from cloudy swelling to widespread necrosis. There is no

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inflammatory reaction. It is usually secondary to some other disease, and the kidney commonly recovers readily when the primary condition heals. A contracted kidney never develops. The injurious substances are bacterial toxins, chemical poisons (uranium, mercury, etc.), and metabolic poisons (as in diabetes and the toxemia of pregnancy). A nephrosis is easily produced experimentally by bacterial or chemical poisons. Experimental uranium nitrate nephritis belongs to this group.

3. The arteriolosclerotic kidney (hypertensive kidney, primary contracted kidney). About three fourths of the cases of primary hypertension show this renal lesion to some extent, but it is seldom far enough advanced to produce any measurable disturbance of renal function. A true primary contracted kidney is rare. The structural change consists in sclerosis of the afferent glomerular arterioles and small arteries with resulting atrophy of glomeruli. After the glomerulus has ceased to function its tubule atrophies and when the atrophy is extensive a contracted kidney results. Nothing definite is known about the etiology of this disease. It has not been produced experimentally.

4. Glomerulonephritis [2] is an inflammation of the kidney, chiefly restricted to the glomeruli. The principal change is a swelling and proliferation of the endothelium resulting in closure of the glomerular capillaries, but there is often a marked proliferation of the cells of the outer layer of Bowman's capsule, giving rise to epithelial crescents, and there may be a notable accumulation of polymorphonuclear leucocytes in the tuft and capsular space. The inflammatory reaction may therefore be described as proliferative and exudative. The closure of all the capillaries of a glomerulus is soon followed by disuse atrophy of its tubule, and it is this tubular atrophy which is largely responsible for the striking gross and microscopic appearances in chronic glomerulonephritis. The microscopic picture is very characteristic and with rare exceptions it is readily distinguished from any other form of renal disease.

Glomerulonephritis includes all the acute and subacute and most of the chronic cases of what is commonly called Bright's disease. Some cases of chronic Bright's disease belong in the group of arteriolosclerotic kidneys. Contracted kidneys in man are usually instances of chronic glomerulonephritis, but a few are of the arteriolosclerotic type.

Glomerulonephritis apparently does not occur in the laboratory animals, but it has been found in cattle and horses.[3] We have not been able to find any literature dealing with nephritis in monkeys.

An excellent review of the literature dealing with experimental nephritis has recently been given by Leiter,[4] to whose paper the reader is referred. It is evident that the only forms of nephritis that have been produced experimentally are exudative interstitial nephritis (corresponding to human pyelonephritis) and nephrosis. A true glomerulonephritis has not been obtained experimentally. The nearest approach to success was by Pernice and Scagliosi[5] who produced a few epithelial crescents in a dog by subcutaneous injections of B. pyocyaneus. Baehr[6] obtained epithelial crescents also by subcutaneous injections of uranium. But in both these instances only a few glomeruli were involved. There was no widespread glomerular inflammation, and the lesions were evidently early, since no glomerular hyalinization or tubular atrophy is mentioned.

*Material and experimental procedure.* Twenty-five rabbits and fourteen young rhesus monkeys were used. Streptococci from human lesions were grown on blood agar slants 24 to 48 hours, and suspensions of the growth were then made in physiologic salt solution. Nonhemolytic streptococci were generally used, since they seemed somewhat less virulent. The suspensions were made fairly uniform in density so that the dosage could be estimated roughly by the number of cubic centimeters injected. A heavy dose for monkeys was from 10 to 15 c.c. of a dense suspension of organisms washed from blood agar slants. The number of slants used varied with the heaviness of the growth, from 3 to 10 slants for a heavy inoculation. Injections were given intravenously and were repeated every few days until a moderate albuminuria developed. The inoculations were discontinued temporarily when the animal showed evidence of toxemia or when a heavy albuminuria was present.

Ten strains of streptococci were used in these experiments. Three of these were hemolytic and seven were nonhemolytic (Streptococcus viridans).

Hemolytic strains: Organism 342 was isolated post-mortem from the blood of a patient dead of septicemia following erysipelas. Organism 4 was obtained at post-mortem from the pericardial exudate of a patient dead of acute rheumatic fever and acute pericar-

ditis. Organism 29 was isolated from the throat of a patient with scarlet fever. The toxin of Organism 29, diluted 1–800, gives a strongly positive Dick test in 0.2 c.c. doses.

Nonhemolytic strains (Streptococcus viridans): Organism 2 was isolated from the blood of a patient with chorea. Organism 1 was obtained from the blood of a patient with acute rheumatic endocarditis. Organism 8 was isolated from the blood of a patient with acute rheumatic fever and endocarditis; and Organism 9 was obtained from the blood of a similar case which also had a pericarditis. Organism 5 was isolated from the exudate of a joint in a case of acute rheumatic fever. Organism 18 was obtained post-mortem from the blood of a patient dead of acute rheumatic fever with acute rheumatic endocarditis; and Organism 11 was obtained post-mortem from the blood and pericardial exudate of a similar case in which a pericarditis also was found.

*Experiments with rabbits.* It seems unnecessary to give these experiments in detail since the results were negative. About 25 rabbits were used and a large number of injections were given some of them. Several animals died of endocarditis. Albuminuria was readily produced. Renal lesions were frequently found at post-mortem, but they were always of the ordinary spontaneous type, that is, lymphocytic interstitial nephritis.

*Experiments with monkeys.* No. 10. Young female. May 21, 1924. Heavy inoculation with Organism 18. Death about 12 hours later from bacteriemia. Kidneys normal.

No. 5. Young female. April 23, 1924. Heavy inoculation with Organism 11. Death about 12 hours later from bacteriemia. Kidneys normal.

No. 6. Young female. April 23, 1924. Urea nitrogen 22.40 mg.; creatinin 1.80 mg. Urine: no albumin. Injected with Organism 8, April 24 and 25. April 26, urine −, albumin +; very sick. April 29, death from bacteriemia. Kidneys greatly swollen and opaque. Microscopic: extensive tubular necrosis, occasional necrotic areas in the glomeruli. No glomerulitis. Diagnosis: severe nephrosis.

No. 4. Young female. March 25, 1924, injected with Organism 11. March 27, second injection. March 28 to April 1, severely ill. April 1, death from bacteriemia. Kidneys slightly cloudy and swollen. No glomerulitis.

No. 12. Small female. June 4, 1924, no albumin. June 10, injected with Organism 29, small dose. June 12, severely ill; albumin +; infection of right eye. June 17, albumin ++; marked swelling and reddening of both lower extremities, especially around the ankles; purulent infection of right eye. June 18, lies prostrate on floor of cage; enormous swelling of ankles. June 21, urea nitrogen 59.3 mg.; creatinin 3.0 mg. Death, June 21. Large quantities of pus in and around ankle joints. Some pus around tendons of right wrist. Swollen kidneys with very opaque cortices. Microscopic: extensive infiltration of the cortex with medium-sized lymphocytes and plasma cells. The exudate is not uniformly distributed but is in irregular areas, spreading out from the small blood vessels. The largest accumulations of lymphoid cells are perivascular but there are large numbers between the tubules and around the glomeruli in the cortex (Fig. 1).

The microscopic appearance corresponds to the acute interstitial nephritis of scarlet fever. It differs from the spontaneous nephritis of the rabbit chiefly in its wide distribution through the cortex. Diagnosis: acute interstitial nephritis.

No. 11. Female. June 4, 1924, no albumin; urea nitrogen 14.0 mg.; creatinin 2.1 mg. Injected with Organism 18. June 2, ? second injection; violent reaction a few hours later. June 9, no albumin; third injection. June 11, no albumin. June 16, no albumin; fourth injection. June 18, albumin +; fifth injection; violent reaction about two hours later. June 19, did not recover from last injection. Death. Kidneys normal.

No. 13. Female. June 21, 1924, no albumin. Small injection of Organism 29. June 24, no albumin; second injection. June 26, third injection. June 27, no albumin. June 30, no albumin; fourth injection. July 2, trace of albumin. Death, July 4. Post-mortem diagnosis: generalized tuberculosis. Kidneys normal.

No. 7. Young male. April 26, 1924, no albumin. April 28, urea nitrogen 12.13 mg.; creatinin 2.14 mg.; injected with Organism 11. April 30, albumin +. Injections were given May 4, 8, 10, 12, and 15. May 11, no albumin. Suppuration developed in one leg at the site of inoculation. Blood chemistry, May 15: urea nitrogen 40. mg.; creatinin 1.8 mg. Death May 15, from bacteriemia. Swollen kidneys. No glomerulitis. Diagnosis: mild nephrosis.

No. 8. Female. May 21, 1924, no albumin; urea nitrogen

22.4 mg. Injected with increasing doses of Organism 9 on May 21, 22, 26, 27, 28, 29, 31, June 2, 4, 6, 9, 11, and 16. June 4, no albumin. Death June 16. Kidneys normal.

No. 14. Female. June 21, 1924, no albumin. Small injection of Organism 29. June 24, no albumin; small injection. June 26, small injection. June 27, trace of albumin. June 30, albumin +. Moderate-sized injections July 9, 11, 14, 15, 17, 22, 26, August 2, 4, 6, 8, 10, 11, 12, 13, 14, 15, 16, 18, and 19. Urine: no albumin July 11, 14, 17, 26, August 4, 12, and 15. August 19, albumin ++. During the last few days evidences of toxemia developed and death occurred at 7 P.M. August 19. Kidneys a little cloudy. Microscopic: occasional necrotic tubules, a few casts. No glomerulitis. Diagnosis: nephrosis of moderate degree.

No. 9. Male. May 21, 1924, no albumin; urea nitrogen 19.6 mg.; injected with Organism 342. May 22, second injection; joints of both lower and the right upper extremities swollen and tender. Injections May 26, 28, 31, and June 2. Albumin +, May 26. No albumin June 2; joints still swollen. Injections June 4, 6, 9, 11, 16, 17, 23, 24, 26, 27, July 2, 3, 9, 11, 14, 15, 17, 18, 22, 26, August 2, 4, 6, 8, 10, 11, 12, 13, 14, 15, 16, 19. No albumin June 17, 23, 26, July 2, 11, 14, 17, 26, August 4, 12, 14, and 16. August 19, albumin ++. Stuporous after August 18. Death August 20. Kidneys about twice normal dimensions; soft hemorrhagic cortices. Microscopic: necrosis of the greater part of the cortices. Blood in all the tubules. An occasional glomerulus shows acute glomerulitis. Diagnosis: severe nephrosis (necrotic type) with very restricted acute glomerulonephritis.

No. 1. Female. Injections with Organism 2, 1st, 4th, 7th, 10th, and 13th days of experiment. No noticeable clinical effect; 13th day, no albuminuria. Injections with Organism 8, 20th, 21st, 24th, and 28th days. No clinical effects; no albuminuria. Injections with Organism 4, 31st, 33d, 35th, 36th, 38th, and 41st days. The animal became very sick after the first injection of Organism 4, and showed some reaction at each subsequent injection. No albuminuria. Injections with Organism 11, 54th, 55th, 66th, 69th, 71st, 73d, 76th, 80th, 89th, 90th, and 92d days. Urine, albumin: 83d day +; 88th +; 92d +; 94th +. Death, 95th day. Blood chemistry, 95th day: urea nitrogen 97.0 mg.; creatinin 2.7 mg.

There were no striking clinical effects from the injections until

Organism 11 was used. After each inoculation with this organism there was a very severe reaction; the animal would vomit and lie on the floor of the cage for several hours. There was a rapid loss of weight. During the last week suppuration developed in one leg at the site of inoculation.

At post-mortem 100 c.c. of clear fluid was found in the peritoneal cavity, but there was no fluid in the other serous cavities and no edema. The liver and the heart showed a faint cloudy swelling. The kidneys were not enlarged but they were very pale and cloudy. Microscopically there was no necrosis of the tubules or glomeruli and no exudate. The glomerular endothelium was swollen, but there was no definite glomerulitis of any kind.

No. 2. Female. Injections with Organism 5. March 1, no albumin. Injections were given March 1, 3, 5, 7, 10, 13, 17, 19, 21, 22, 26, 27, 30, and 31. April 1, no albumin. Injections April 2, 4, 5, 7, 10, 23, 24, 26, 28, and 30. May 1, trace of albumin; marked loss of weight. There was a slight reaction after each injection during March and April but there were no violent reactions.

Injections May 3, 5, 8, 10, 12, 15, 19. Urine, May 7: albumin++; May 11, albumin +++; May 19, albumin +++; May 22, albumin +++. Blood chemistry May 19: urea nitrogen 60.7 mg.; creatinin 2.1 mg. During May the reaction following each injection was more severe and emaciation became extreme.

Very small injections were given June 2 and 4. These were followed by severe reactions. Urine: June 2, albumin +++; June 6, albumin +++. Blood chemistry June 11: urea nitrogen 36.4 mg.; creatinin 2.5 mg.; June 16, urea nitrogen 49.3 mg.; creatinin 3.0 mg. Death June 16.

At post-mortem there is marked general emaciation. There is no edema and no fluid in the serous cavities. The heart and liver are faintly cloudy. The heart is of normal size.

The kidneys are moderately enlarged and their capsules are slightly adherent. The surfaces are finely granular. The cortices are opaque in appearance and no markings are visible. On microscopic examination most of the tubules show a definite reduction in size, but those connected with badly damaged glomeruli show a pronounced atrophy (Figs. 2, 3, 4, 5). Some of the larger arteries of the medulla show an acute suppurative inflammation which has resulted in aneurismic dilatations. The entire circumference of a

segment of the artery is involved. The walls of the diseased portions are densely infiltrated with polymorphonuclear and large mononuclear leucocytes. There is a striking resemblance to the lesions of periarteritis nodosa. A similar suppurative arteritis has been observed by us in subacute bacterial endocarditis. The diseased arteries in this kidney are responsible for some of the cortical atrophy; but the tubules that show a pronounced atrophy may be traced to glomeruli with closed capillaries (Figs. 2, 3, 4, 5). There is no necrosis of the tubular epithelium.

The glomeruli show very striking changes. The most conspicuous lesion is hyperplasia of the outer layer of Bowman's capsule with the formation of *epithelial crescents* (Figs. 6, 7, 8). This is often called extracapillary glomerulitis. It is found in 5 to 10 per cent of the glomeruli in different parts of the kidney. The crescent compresses the glomerulus and prevents the circulation of blood through its capillaries. A disuse atrophy of the glomerulus and its associated tubule follows. This type of lesion is frequently seen in human acute glomerulonephritis.

The most common glomerular lesion is *fusion between the glomerulus and the outer layer of Bowman's capsule* (Figs. 4, 5, 9, 10, 11). This results in partial or complete obliteration of the capsular space. In many of the glomeruli there is *swelling of the endothelium with closure of some of the capillaries* (Figs. 5, 8, 9, 10, 11, 12). Polymorphonuclear leucocytes are sometimes found in these closed capillaries (Fig. 11) and the swollen cells may show hyaline changes in their cytoplasm (Fig. 12). A true proliferative glomerulitis (increase in number of endothelial cells) is not a prominent feature. The proliferative lesion, it will be recalled, is the most common type in human glomerulonephritis. An occasional glomerulus shows large numbers of polymorphonuclear leucocytes in its capillaries and in the capsular space (Fig. 13). This is called *exudative glomerulitis*.

To summarize: Over half of the glomeruli show some form of injury which tends to interfere with their function. There are numerous instances of fusion between the tuft and the capsule of Bowman, and many glomeruli have a part of the capillary network occluded by swollen endothelial cells. Epithelial crescents are frequent and there are a few examples of exudative glomerulitis. The tubules belonging to the occluded glomeruli show a well-defined atrophy. The similarity to human glomerulonephritis is striking.

The lesion may be classified as an early stage of chronic glomerulonephritis. A convenient histologic distinction between acute and chronic glomerulonephritis is the presence of a well-defined tubular atrophy in the latter. The increase of urea nitrogen in the blood indicates a moderate degree of renal insufficiency, which is consistent with the histologic appearances.

No. 3. Large male. This experiment will not be reported in full in this paper since the monkey is still living. The experiment was begun March 1, 1924. Albuminuria has been present since May, 1924, and it has been severe since August. Recently (January, 1925) there has been a large amount of blood in the urine. Small injections, one to four weeks apart, are sufficient to keep up the severe albuminuria. The kidneys can still concentrate well, however, and the blood metabolites are not increased. There is no edema. The blood pressure is not known. The type of renal lesion present cannot be determined with certainty in the living animal.

## Discussion

The intravenous injection of streptococci into fourteen monkeys produced severe renal lesions in five. There were two cases of severe nephrosis (Nos. 6 and 9), one of acute insterstitial nephritis (No. 12), one of glomerulonephritis (No. 2), and one of a nature not yet determined (No. 3), since the animal is still living. In the other 9 monkeys the organisms caused death without producing any serious renal injury. It is known that streptococcic infections in man may result, also, in glomerulonephritis, nephrosis, interstitial nephritis or an insignificant renal injury. No investigator has offered a satisfactory explanation of this varying effect of streptococci on the kidneys. It is known that in some cases at least of glomerulonephritis the endothelial cells of the glomerulus ingest the bacteria, and then undergo swelling and proliferate as a result; but bacteriemia usually causes nephrosis, not glomerulitis. There is some necessary factor other than the presence of bacteria in the blood stream that determines whether a glomerulitis will result. It is not known whether soluble bacterial toxins, without the bodies of the bacteria, can cause glomerulitis.

Three hypotheses may be offered to explain the origin of glomerulonephritis. (1) There is a specific etiologic agent — some special

strain of streptococci. (2) Many types of streptococci may produce the disease but only certain individuals are susceptible. (3) There must be repeated injury to the glomerular endothelium. Individual susceptibility and the strain of the infecting organism are perhaps not important. Our results thus far do not justify any conclusion as to which of these hypotheses is correct; but we are proceeding on the theory of repeated glomerular injury.

It may be objected fairly that our single successful experiment (No. 2) does not prove that glomerulonephritis is caused by streptococci, since it is possible that the disease may have developed during the course of the experiment independently of the experimental procedure. Other positive results must be obtained to establish the etiology of glomerulonephritis with certainty, but we feel that the solution of the problem is definitely nearer. An animal has been found in which the disease may develop, and there is at least a strong probability that the organisms injected produced the disease.

## Conclusions

Intravenous injections in rabbits of several strains of streptococci from human sources produced in several instances nephritis of the spontaneous type (lymphocytic interstitial nephritis) but did not result in glomerulonephritis.

Similar injections in monkeys resulted in two cases of severe nephrosis, one of acute interstitial nephritis, one of glomerulonephritis, and one of a type not yet determined.

The case of glomerulonephritis was characterized by epithelial crescents, fusion of glomerular lobules to the capsule of Bowman, swelling of the endothelium with occlusion of the capillaries, and atrophy of tubules associated with occluded glomeruli.

Glomerulonephritis has been produced in one monkey by intravenous injections of streptococci.

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## DESCRIPTION OF PLATES XLV–XLVIII

### PLATE XLV

FIG. 1. Monkey No. 12. Drawing. Acute interstitial nephritis. Large numbers of mononuclear leucocytes are found, widely distributed between the tubules in the cortex. They are most numerous around the small veins.

FIG. 2. Monkey No. 2. Photomicrograph, low magnification. Injured glomeruli with atrophy of their associated tubules.

FIG. 3. Monkey No. 2. Photomicrograph. Part of the field of Fig. 2 under higher magnification. One glomerulus is almost completely occluded by closure of its capillaries and fusion of the lobules to the capsule of Bowman. The glomerulus with the open capsular space shows collapse of its capillaries, and a thin epithelial crescent to which the glomerular lobules are partly adherent. The tubules show a marked atrophy.

### PLATE XLVI

FIG. 4. Monkey No. 2. Photomicrograph. In about half of the glomerulus the capillaries are closed by swollen endothelial cells, and this part is fused to a thin epithelial crescent. The tubule is markedly atrophied.

FIG. 5. Monkey No. 2. Photomicrograph. The capillaries are all closed by swollen endothelial cells and the capsular space is largely obliterated. The tubule is markedly atrophic.

FIG. 6. Monkey No. 2. Photomicrograph. A large epithelial crescent. The upper right-hand portion of the glomerulus shows occlusion of its capillaries, and it has become fused with the crescent and the capsule of Bowman.

FIG. 7. Monkey No. 2. An epithelial crescent. The glomerulus is anemic.

### PLATE XLVII

FIG. 8. Monkey No. 2. Photomicrograph. Higher magnification of a glomerulus shown in Fig. 10. There is a small epithelial crescent with fusion to the glomerulus in several places. In the central part of the glomerulus the capillaries are closed and there is some disintegration of the swollen endothelial cells.

FIG. 9. Monkey No. 2. Photomicrograph. Glomerulus showing extensive closure of capillaries and fusion to the capsular epithelium.

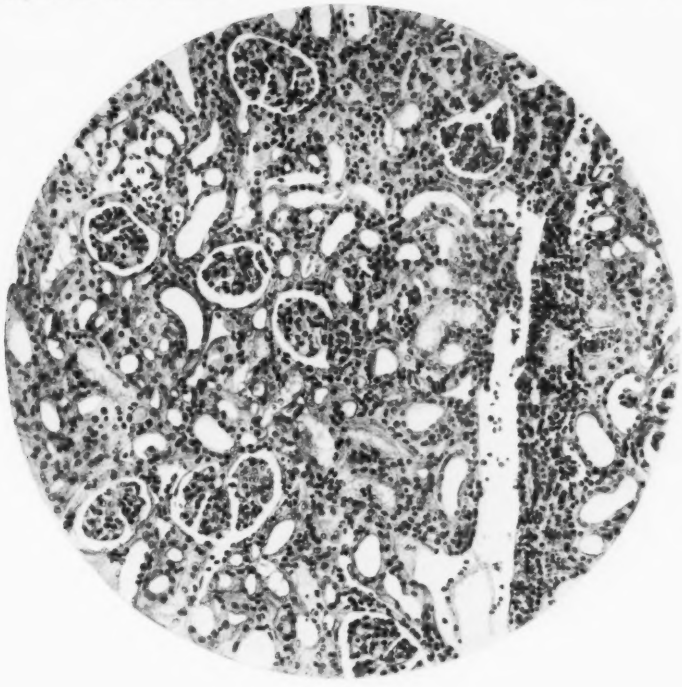
FIG. 10. Monkey No. 2. Photomicrograph. Two glomeruli showing inflammatory reactions. The left-hand glomerulus is shown under higher magnification in Fig. 8; the right in Fig. 11.

FIG. 11. Drawing of the lower half of the glomerulus shown in the left-hand portion of Fig. 10. There has been extensive proliferation of the endothelium with occlusion of the capillaries. In the upper left-hand portion several polymorphonuclear leucocytes are seen imprisoned in closed capillaries. This appearance is frequently seen in the proliferative type of human glomerulonephritis.

FIG. 12. Monkey No. 2. Photomicrograph. An enlarged glomerulus showing extensive swelling of the endothelium with capillary occlusion. A number of polymorphonuclear leucocytes are seen.

## PLATE XLVIII

FIG. 13. Monkey No. 2. Photomicrograph. An example of exudative glomerulitis. There are a large number of polymorphonuclear leucocytes in the capsular space.



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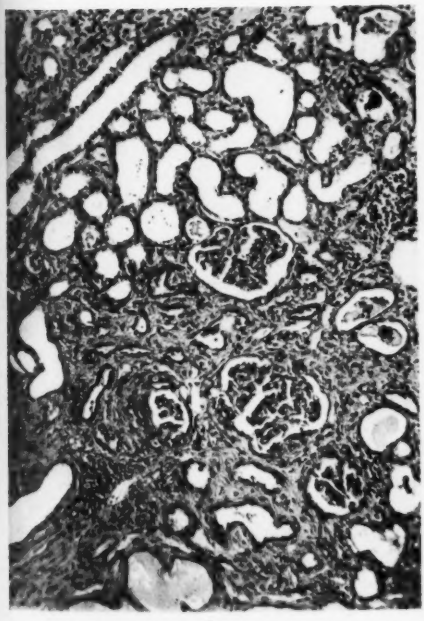

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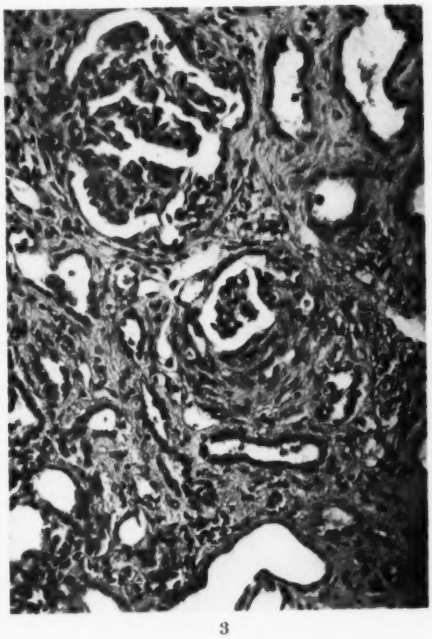

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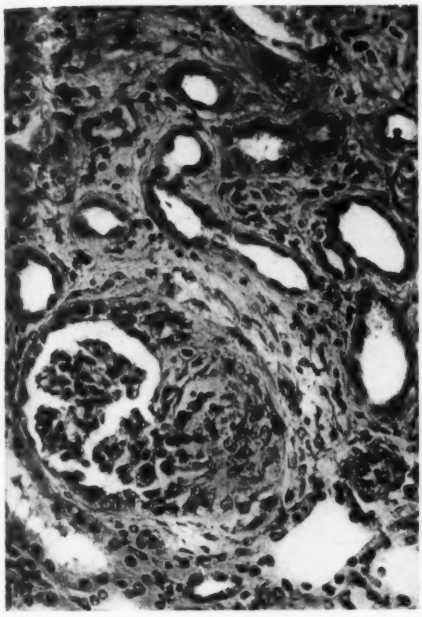
Bell, Clawson and Hartzell

Experimental Glomerulonephritis



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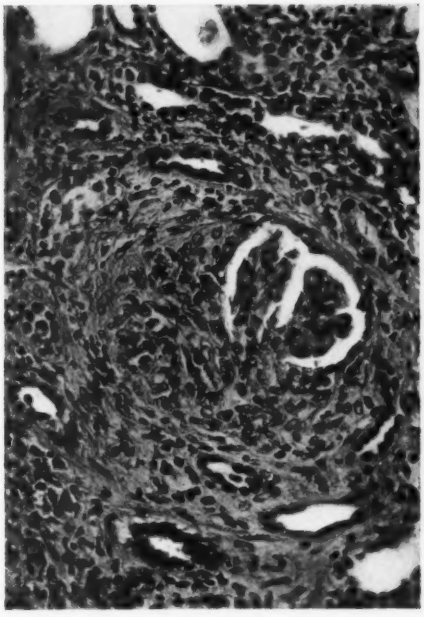

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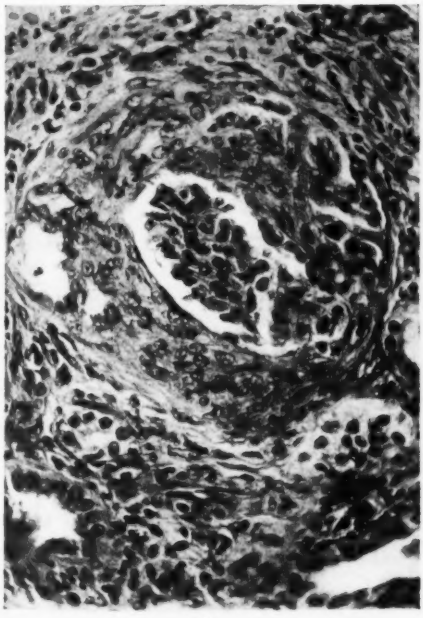

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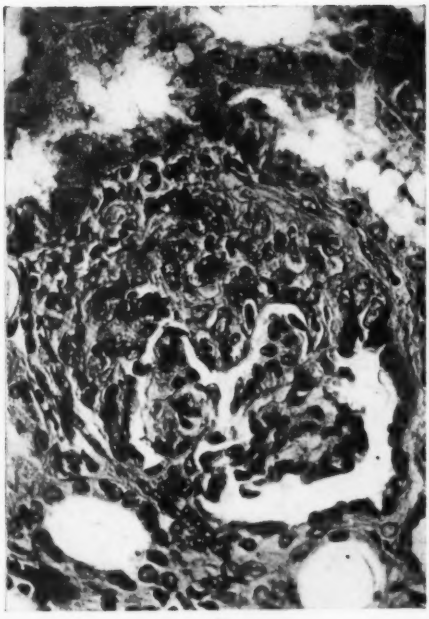

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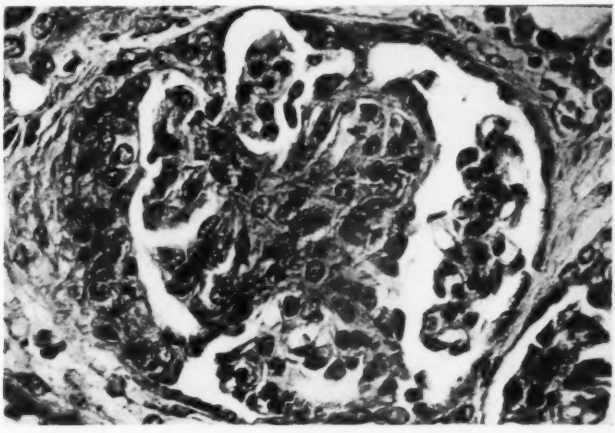

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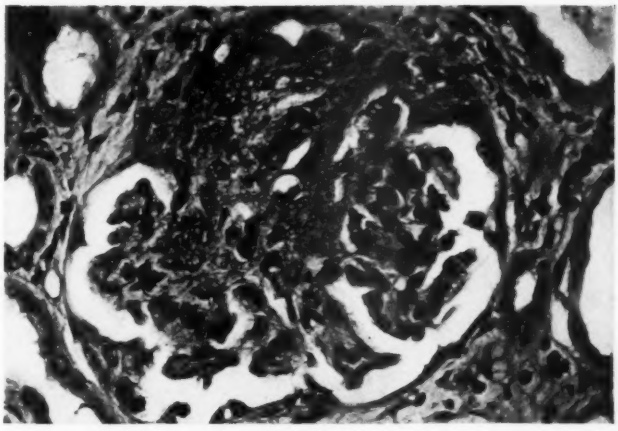

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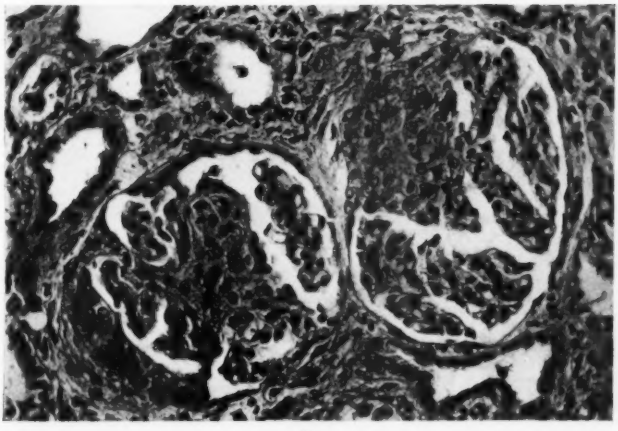

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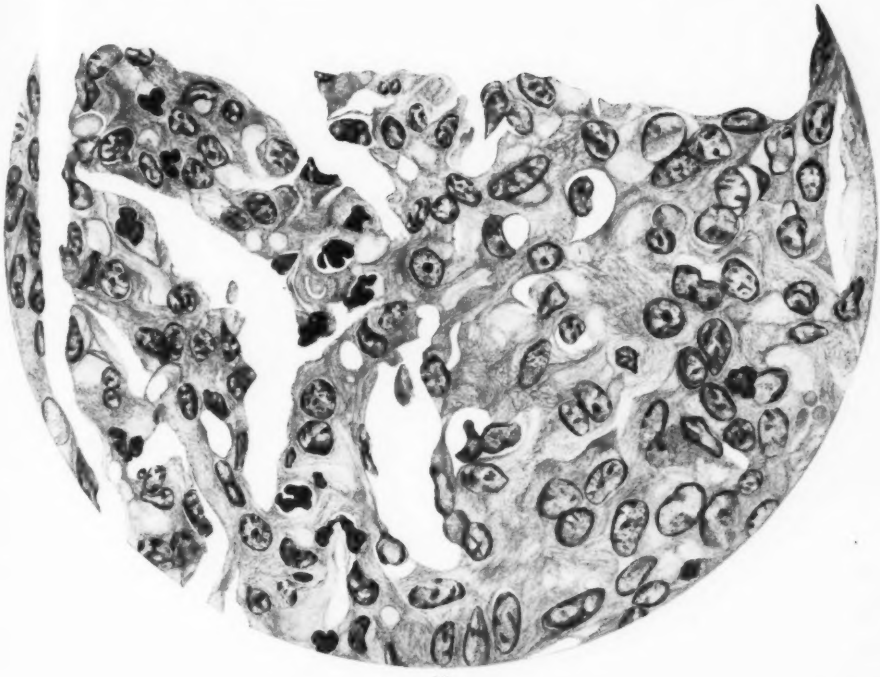

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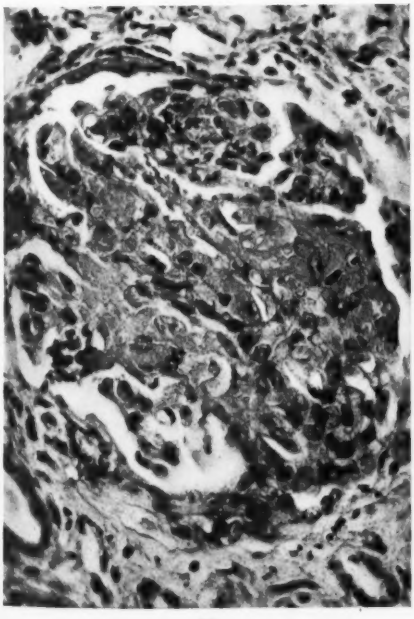

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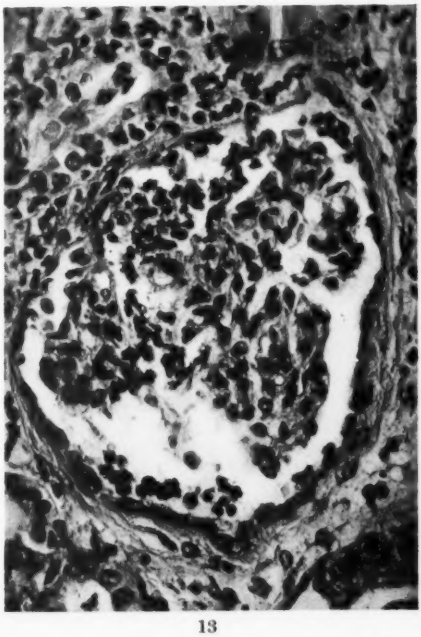

13

Bell, Clawson and Hartzell

Experimental Glomerulonephritis

# THE FIBROMA OF THE ACOUSTIC NERVE*

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The cerebellopontile angle is an anatomical site in which certain neoplasms may develop, the glioma, the dural endothelioma and the acoustic nerve fibroma comprising the very small group of tumors (except for a rare metastatic growth) which have been found in this location. In 1917 Dr. Harvey Cushing[1] in his monograph "Tumors of the Nervus Acusticus" directed attention to the acoustic nerve tumor as a clinical and pathological entity. His own studies and those of Councilman, Mallory and Goodpasture lead also to the differentiation of a histologic picture characteristic of most tumors in the aforementioned locality, their origin usually being in the sheath of the eighth cranial nerve.

The greater percentage of acoustic tumors are unilateral. When bilateral they are usually an expression of a widespread affection of nerve sheaths, commonly known as von Recklinghausen's disease. Berggrün,[1] Raymond,[1] and Bassoe and Nuzum[2] have reported instances of bilateral tumors and others are on record. Dr. Cushing had none in his series of ninety-one acoustic tumors. This paper concerns three cases of cerebellopontile angle tumors which have come to autopsy in the pathological department of the Albany Medical College during the past three years. In one of them these neoplasms were bilateral.

It will not be attempted here to discuss with much elaboration the clinical aspects of these three patients. Their histories will be given in all available detail but the reader will be left to analyze for himself much of the clinical data. To the pathologist the anatomical and histologic characters of these tumors and their secondary effects are of greatest interest and furnish a rational explanation of the usual symptom complex. That tumefactions in the cerebellopontile recess, of eighth nerve origin, are productive as a rule of a pathognomonic syndrome is generally recognized; hence a brief presentation of that syndrome is apropos.

* Received for publication February 18, 1925.

"The acoustic tumors," Dr. Cushing states, "owing to the characteristic chronology of their symptoms, may as a rule be sharply distinguished from all other tumors of the cerebellopontile angle.... The symptomatic progress of the average acoustic tumor occurs more or less in the following stages: first, the auditory and labyrinthine manifestations; second, the occipito-frontal pains with suboccipital discomfort; third, the incoördination and instability of cerebellar origin; fourth, the evidences of involvement of adjacent cerebral nerves; fifth, the indications of an increase in intracranial tension with a choked disc and its consequences; sixth, dysarthria, dysphagia, and finally cerebellar crises and respiratory difficulties." For a bibliography of the condition the reader is referred to Dr. Cushing's work. Nothing of significance has been published since.

*Case I.* Hospital number 81547. Mrs. L. A., white, aged 46 years, admitted to the Albany Hospital, October 26, 1921, complaining of gradual loss of sight and hearing over a period of one and a half years, and difficulty in walking.

History: her general health has been good until eight years ago, when her head began to be spasmodically drawn to one side. Attacks of fainting during the first three weeks of illness and vertigo daily. Many attacks of blindness lasting from two to seven minutes and a general impairment of sight for one and a half years. Hearing has been poor past year with total deafness and tinnitus last eight months. Great difficulty in forming sentences although her memory seems good. Last two months dysphagia and marked dyspnoea. Lately her "flesh feels creepy."

Physical examination: The important findings are neurological. Eyes: O. D., pupil 4.5 mm., regular, movements slow. Media clear, disc swollen with edema, 8 diopters. Vessels large and prominent. O. S., pupil regular, 4.5 mm. diam., movements slow. Media clear. Disc swollen 8 diopters. Vertical pseudonystagmus when looking up. Diagnosis from ocular findings, choked discs, very great intracranial pressure.

X-ray examination: skull is unusually thick. The picture is unsatisfactory on account of the movements of the patient.

Summary of findings of Dr. LaSalle Archambault: Ocular excursion is restricted in all directions, but more markedly so upward and to the left. The pupils react actively to light. Facial and hypoglossal nerves negative. No paralysis in arms. Grasp excellent in both hands, but there is some evidence of asynergy and adiadokokinesis. There are similar findings in the legs. All reflexes more active on right side of body. A doubtful Babinski on right side. Many tests not attempted, owing to the marked deafness of the patient, which renders coöperation impossible. Impression: a lesion (possibly tumor) involving the dorsal segment of the pons Varolii at the junction of the superior cerebellar peduncle, the corpora quadrigemina and the posterior longitudinal bundle.

A decompression was advised but not done. She died April 15, 1922.

Autopsy was performed two hours post-mortem. The body shows much emaciation. On opening cranial vault and dura there is seen

a great increase of clear subarachnoid fluid. Cerebral cortex edematous and convolutions slightly flattened. On severing the tentorium cerebelli and lifting the brain upward and backward, two roughly oval, somewhat lobulated tumor masses are found lying one along each side of the pons and medulla and extending upward and forward to form indentations in the under surface of the lateral lobes of the cerebellum. The tumors have produced tremendous pressure upon and distortion of the brachia conjunctiva and the pons, so that the basilar artery takes a winding course over the structures normally in the midline. The masses have a rather smooth, grayish-pink surface and their attachments comprise chiefly their blood vessels and accompanying prolongations of arachnoid. On section each mass shows a grayish striated, very tough structure, not unlike a leiomyoma or dense fibroma. The eighth nerves are identified with difficulty, having broken away from the tumors to which they had apparently been attached. Gross examination of the other cranial nerves fails to reveal any other evidence of neurofibromatosis.

On section of the cervical cord, the central canal is found dilated to a diameter of two millimeters.

*Microscopic examination.* Sections of both tumors were stained with haematoxylin and eosin, phosphotungstic acid-haematoxylin, Mallory's aniline blue, and by Van Gieson's method.

The tumors are surrounded by a thin connective tissue capsule in which are dilated lymph and blood vessels. Predominating in the picture are areas of fibrous tissue running in irregular bands with a definite tendency of the nuclei of the cells to be arranged in a regimental manner. The fibrous zones alternate with smaller areas of loose edematous reticular tissue. All sections of the tumors are very vascular and in and about the reticular areas are many thrombosed vessels, some of which show hyaline degeneration of their walls. No neuroglia cells or fibers can be demonstrated in many sections examined. There is a general uniformity of size and staining of the spindle cells of the tumor in most sections, but in others the nuclei are large and deeply staining, like young cells and more rapidly growing. Mitotic figures are not found, however.

*Case II.* Hospital number 86470. Miss J. B., aged 22 years, entered Albany Hospital, September 21, 1922, complaining of double and blurred vision, transitory blindness and dizziness. Present illness began in 1921, with disturbance of vision and marked vertigo on change of position. For an indefinite period she has awakened each morning with occipital headache. Vision good until Sep-

tember 1, 1921, when there were sudden attacks of diplopia with blindness of two or three minutes' duration. Vision always worse in morning. No loss of color vision.

Neurological examination: N. I., no disturbance of smell; N. II, O. D., pupil 5 mm., regular and active. Media clear. Great elevation of disc, plus four diopters. Vessels of fundus of irregular caliber and in many places obscured. A few superficial retinal hemorrhages. Vision 20/20. Partial ptosis of lid. At rest pupils are 20° convergent. Coarse rapid lateral and vertical nystagmus and slight exophthalmus. O. S., has same fundus changes as O. D. Some muscle jerking and internal strabismus. Both pupils react to light and accommodation consensually. N. V, no motor or sensory disturbance. N. VII, normal. N. VIII, no tinnitus; vertigo on change of position. Air and bone conduction not tested. NN. IX, X, XI, XII are normal.

Great mental irritability during the past year. Well oriented as to time and place. Romberg, slight swaying. X-ray examination of skull reveals no evidence of tumor. Spinal fluid, September 19, 1922, globulin 4 plus, cells 8. Spinal fluid, October 18, 1922, globulin 3 plus, cells 6.

Diagnosis: unlocalized brain tumor.

Operation performed by Dr. Arthur H. Stein. A subtemporal decompression was done. On opening dura a considerable quantity of clear fluid escaped. There was considerable hemorrhage which was very hard to control. No gross evidence of tumor in cortex. Some of the more marked symptoms appeared to be relieved by the operation. On seventh day patient suddenly collapsed and from then on rapidly grew worse. Previous symptoms became more marked and a hernia cerebri very pronounced at the site of operation. There were much drowsiness, nausea and vomiting. Spinal fluid was under enormous pressure and some relief was gained by lumbar punctures. Patient gradually became weaker and more emaciated, due to the fact that she was unable to retain nourishment. Cerebral type of vomiting. Complete left-sided paralysis developed and lasted six weeks. Her condition gradually became worse with death on December 9, 1922.

Autopsy two hours post-mortem. There is a distinct bulging on the side of the head within the limits of the operative incision. This area is soft and fluctuant and scalp is adherent. The brain substance is bulging through the operative opening, being held by the temporal fascia and muscle.

On removal of the skull cap, the dura appears normal, except that it is so closely adherent to herniated area of the brain that it has to be removed with the brain. There is no edema or congestion of the blood vessels. The cerebro-spinal fluid is normal in amount and is perfectly clear. The longitudinal and transverse sinuses appear normal. The superior surface of the brain is symmetrical, except for the herniated area, which measures 6 × 8 cm. and projects 2 cm. above the general contour of the brain. This area is soft and spongy. The consistency of the brain is quite uniform over the entire convexity of both hemispheres. On removing the brain, the tentorium cerebelli is quite taut, especially on the left side, although there is no

bulging, but when it is cut on the left side, a yellowish tumor mass immediately protrudes through the opening, to which two or three good-sized blood vessels are running from the arachnoid.

The Inferior Surface of the Brain. There is a distinct cerebellar pressure cone. The under surface of the temporal lobes show slight roughening, as though adherent to the membranes. A rather soft and rubbery tumor mass occupies the region of the middle cerebellar peduncle, just anterior and superior to the left cerebellopontile angle. The visible portion of the tumor measures 3 × 3 × 2.5 cm. and on palpation extends back into the cerebellum into the amygdala and biventral lobe about 2 cm., so that from the inferior only a small portion of the lateral part of the lobuli centralis can be seen. The tonsil cannot be seen at all. It cannot be determined from a general examination whether the tumor mass is invaginated into the cerebellum or is an outgrowth from it. It occupies nearly the whole supero-inferior thickness of the cerebellum. It is invaginated into the pons, medulla and olives on the left side, distorting them markedly. The fifth nerve passes out over the tumor, between it and the brain stem and is pushed upward. There has apparently been no compression of the nerve. The entire brain stem has been pushed anteriorly and to the right. The tumor mass itself is roughly oval in shape, yellowish in color, smooth in outline and slightly firmer than the surrounding brain tissue. It is underneath the pia. There is much congestion of superficial blood vessels of the tumor and of the cerebellum.

*Description of the brain after hardening in 4 per cent formaldehyde.* The anterior tip of the right temporal lobe on its inferior surface has a moth-eaten appearance. There are numerous small areas in which there is an indentation of the cortex. The larger of these areas measures 0.5 cm. in diameter and extends down into the central white matter. There are numerous other smaller areas similar to the one described, but none of these extends through the cortex.

Frontal section at the level of the optic chiasm reveals considerable dilatation of the lateral and third ventricles. The recessus opticus is considerably dilated. The inferior and posterior horns of the lateral ventricles are involved in the dilatation.

There is considerable distortion of the convolutions on the right side at the bottom of the Sylvian fissure, due to the decompression.

The central white matter adjacent is much softened and has a pinkish tinge. At the level of the anterior commissure, this softened focus comes within 2 mm. of the dilated lateral ventricle.

On section there is seen to be marked asymmetry of the cerebellar hemispheres, the left being much larger. The left inferior cerebellopontile angle and the adjacent portion of the left cerebellar hemisphere are occupied by an encapsulated tumor mass measuring 3 × 5 cm. in its greatest diameters.

The tumor mass is only slightly adherent to the cerebellum but instead is invaginated into the left cerebellar hemisphere, pushing the cerebellar structures aside and compressing them. The tumor is surrounded by a delicate connective tissue capsule from the outer surface of which run fibrous tags attaching the tumor to the adjacent cerebellar surface and to the pons. The external surface of the tumor is quite irregular in contour, due to numerous hard elevations averaging 0.5 cm. in diameter. There are numerous dilated blood vessels on the external surface. The cut surface of the tumor presents a mottled, slightly gelatinous appearance. Numerous foci of hemorrhage and degeneration are scattered throughout.

There has been produced a deep excavation where the tumor has encroached into the left cerebellar hemisphere.

The cochlear division of the eighth nerve is much enlarged on the left side and runs on the superior surface of the tumor mass. It is very adherent to the tumor and apparently is lost in the substance of the tumor. The other cranial nerves show nothing noteworthy.

*Microscopic examination.* The sections are of a rather vascular neoplasm, composed of fasciculi of fibroblasts arranged in irregular bands and whorls, with in places a well-defined tendency toward regimentation of the nuclei, and of a loose, reticular tissue varying in amount from small isolated patches to predominance in some sections. As a whole, the reticular tissue is much less in amount than the fibrous tissue.

The fibroblasts have usually vesicular ovoid nuclei with one to three nucleoli, but there is variation with the nucleus being larger, more chromatic and irregular in outline. Fibroglia fibrils are conspicuous in phosphotungstic acid-hematin preparations and collagen fibrils form in several a fair amount of intercellular substance. Edema is very marked in the capsule and in large areas of tumor,

the fibroblastic tumor cells becoming very hydropic, so that little or no cytoplasm is in evidence. Contributing to the vacuolated "reticular" tissue is a moderate amount of fat and lipoid within cells of many shapes, but practically all of which can be traced back to the spindle tumor cells. Thrombosis and hyalinosis of blood vessels in and about the rarefied areas suggest the cause of the hydropic and fatty changes so much in evidence. Many of the blood vessels are closely packed with leucocytes, and there is perithelial or adventitial migration, chiefly of polymorphonuclear neutrophiles, about several of the smaller vessels.

*Case III.* Hospital number 94868. Mrs. L. J., a white American housewife, aged 44 years. Chief complaint is pain in the epigastrium, spine, and occipital regions. She has difficulty in recognizing distant objects with right eye and especially difficulty in seeing when fatigued. Hears poorly with right ear. Occasional attacks of tinnitus. Recently there has been pain in back of head and along entire spine, associated with a spastic condition of the muscles of the right side of face and right leg. In 1922 she began to be ailing, with severe left frontal headaches which were worse at night. Marked tinnitus aurium. In 1923 she had several attacks of faintness. Frequent severe attacks of abdominal pain requiring morphia. In November 1923, occipital pain developed and during the most severe attacks power to use arms and legs was lost. During one such attack the right angle of the mouth was drawn upward with twitching of all the muscles of the right side of the face and right leg. Profuse sweating of the right arm.

Physical examination: patient rather nervous and talkative. Both pupils react to accommodation. Bilateral horizontal nystagmus. Restricted movements of left eye toward left and of right eye toward right. Upward excursion of right eye limited. Bilateral vertical nystagmus but no downward limitation of motion. Some lagging of left eyelid. Bilateral choked discs. Optical fields suggest nasal and inferior contraction. Tongue deviates slightly towards left. Left angle of mouth elevated more than the right when upper lip is raised. Uvula deviates slightly to the right. Pain on pressure over the occipital nerve on the left side (Bing's sign). Upper extremities: reflexes slightly more active on the left and grasp is somewhat firmer on the left. Abdominal and ankle reflexes not elicited. No motor losses.

X-ray report, January 10, 1924: "No definite evidence of intracranial tumor."

January 15, 1924, hearing test:

| | 50 | 200 | 2000 |
|---|---|---|---|
| Right ear | − | − | − |
| Left ear | + | + | + |

First operation performed January 15, 1924, by Dr. Arthur Stein. The skull was trephined approximately 0.5 cm. below and to the right of the occipital protuberance. Dura was opened by a crucial incision exposing the cerebellum. The right lobe of the cerebellum was somewhat more prominent in the incision and showed venous congestion. To palpation the right lobe was more firm and

resistant, but no tumor mass could be felt with certainty. The patient's condition was such at this stage of the operation that it was decided to close the incision and do a second stage operation at a later date. A diagnosis was made of a right pontine tumor.

Second operation was done January 24, 1924. The old incision was opened and cerebellum uncovered. The right lobe of cerebellum showed marked bulging with evidence of necrosis of the outer layer. An attempt was made to elevate the right lobe of the cerebellum and reach the tumor beneath, but this could not be accomplished. The patient's condition would not permit further exploration, so the incision was closed and the patient was returned to the ward, where she died January 31, 1924.

Permission was obtained to explore the brain through the operative incision. This was done and a tumor approximately 3 × 2.5 × 2.5 cms. was found, just above and anterior to the foramen magnum, and slightly to the right side. It was eroding the bone and had apparently involved the dura. The tumor mass was quite hard.

Gross description: The cerebellum, medulla and pons were removed, together with a tumor from the posterior fossa, through an occipital flap made for previous operations. The cerebellum is greatly lacerated, so that the connections of the tumor cannot be made out. The tumor is roughly spherical, faintly lobulated, measuring 4.2 × 3.7 × 2.5 cms., inclosed in a delicate but tough fibrous capsule, which is interrupted over a very ragged surface 2.2 cms. in diameter, suggesting a point of attachment. It weighs 17 grams. The tumor is somewhat soft and homogeneous. Cut surface is smooth, rather translucent and bulges slightly from the capsule. On close inspection, fine fibrous strands are made out, frequently interrupted by irregular spots of opaque yellow. Many punctate hemorrhages mottle the surface.

*Microscopic examination.* The tumor is surrounded by a thin fibrous capsule, in which are blood and lymph vessels. The neoplasm is essentially fibrous throughout, with fairly abundant vascular supply. Large foci have undergone anemic necrosis, due to thrombosis of many large thin-walled blood vessels, which are also greatly dilated. The type cell is spindle and of varying size, from narrow, typically mature, ordinary fibroblasts, to larger, shorter cells, with nucleus of deeper tinctorial reactions and of much variety as to size and shape. No mitotic figures are found. The tumor cells are arranged in wavy bands, large tracts and in coarse whorls, the nuclei in places being arranged in so-called regimental or palisade manner. Fibroglia

fibrils are well demonstrated by specific connective tissue stains. There are many portions, usually adjacent to necrotic tissue, which present a picture of less marked degeneration, but with more vacuolation of cytoplasm and the formation of large, clear spaces quite like the "reticular" zones described in Cases I and II. In the vicinity of foci of necrosis or degeneration are groups of large, pale phagocytes, filled with lipoid.

## Discussion

The acoustic neurofibroma had possibly been described as early as 1777 by Sandifort of Leyden,[1] but it is not certain absolutely that he was not dealing with a primary meningeal tumor. Jean Cruveilhier[3] in 1835 gave a masterly description of the clinical and gross pathologic findings in a true case, with drawings which leave no doubt as to the nature of the growth. Toynbee[4] in 1853 and Stevens[5] in 1879, published observations of undoubted acoustic tumors. Sternberg[1] in 1900 reported four cases of his own and reviewed the literature. About this time experimental studies gave more accurate means toward the detection of cerebellar and other posterior brain lesions, so that from then on acoustic or "recess" tumors were more frequently recognized. For a complete bibliography to 1917 one should consult Dr. Cushing's monograph.

In a series of 468 verified brain tumors observed by Cushing to February 1, 1917, 134 were lesions in the posterior fossa involving the mid and hind brain; 56 of these were extra-cerebellar tumors and of these 30 came from the acoustic nerve. Six per cent, then, of all brain tumors were derived from the eighth cranial nerve. In a personal communication to the writer, Dr. Cushing brought the cases up to a very recent date; in a total of 1035 verified brain tumors there were 91 of these acoustic tumors or 8.8 per cent.

In Cushing's first series, eighteen were in females and twelve in males. The figures of Henschen[6] and Bruns,[1] on the other hand, show a slight predominance of males. The three cases here recorded were all in women. It is thus seen that there is no very definite sex predisposition to these lesions.

The first symptoms of acoustic tumor are often not remembered by the patient and hence the real date of onset of the disease cannot be told with accuracy. In Cushing's first series he found that 34

years was the average approximate age of onset. Our three cases were respectively 46, 44, and 22 years old at the time of entrance to the hospital for the alleviation of distressing symptoms.

Bilaterality in acoustic tumors is very rare. Dr. Cushing has had no instance in his large experience, although he has suspected it in several patients. Berggrün [1] and Bassoe and Nuzum [2] have reported each a case, the former in a child of eleven years with inherited generalized neurofibromatosis, the latter in an adult with neurofibromatosis. Raymond's [1] case apparently was without generalized nerve involvement as was our Albany case. Henschen [6] in 1915 compiled a large series of acoustic tumors, 245 unilateral and 24 verified bilateral tumors, the latter usually but not always identified with widespread neurofibromatosis.

The acoustic neurofibroma is said to originate from the perineurium of this nerve and usually in the distal portion of the nerve within the auditory canal. There may result through pressure absorption a dilatation of the porus acusticus internus. In the three Albany cases there was no helpful X-ray finding, but they were not studied sufficiently from this point of view to rule out changes in the porus. The post-mortem examinations also did not pay specific attention to this detail.

The cerebellopontile angle tumors have been known by many names, the long list only accentuating the lack of unanimity among pathologists as to the origin of the type cell and the significance of various other cells and their processes. Some of the terms found in the literature include the following: steatoma, fibroma, sarcoma, neuroma, glioma, endothelioma, sarcoma fusicellulare, neuroma fasciculare, neurofibroma, endotheliosarcoma, gliomes polymorphes angiomateux, neurinoma, and fibroblastoma.

Throughout this list there runs fairly consistently a vein of recognition of the outstanding feature of these tumors, i.e., their essentially fibrous nature. That there should be at times confusion with the dural endothelioma is easily understood because of the extracerebellar position and the similar texture of the two kinds of tumors. Mallory [7] only a few years ago concluded that the dural endothelioma was really a fibroblastoma because the tumor cells were capable of forming fibroglia and elastic and collagen fibrils. An association of true meningeal tumors with acoustic tumors has been observed by several writers since Wishart [8] first noted it in 1822.

Glia fibrils are known to occur in these tumors at times but usually they are hard to find. Their presence would seem to be largely accidental and more in the nature of inclusions or congenital glial rests, although in view of their occasionally having been seen it is perhaps natural that the diagnosis of glioma or gliosarcoma has been made.

What, then, is the characteristic appearance of these acoustic tumors? They are encapsulated, somewhat lobulated, more or less vascular fibrous tumors which vary in size from a few millimeters to as much as seven centimeters. The blood vessels are thin walled and prone to small hemorrhages so that hemosiderin deposits may be present. The vessels are also inclined to thrombosis and hyalin change with consequent focal necrosis, lipoid phagocytosis, and degenerations, the latter including probably the so-called reticular tissue. The type cell is a narrow spindle cell of fibroblast morphology but with slight variation from the normal tinctorial fibroglia and collagen reactions. The cells are arranged in interlacing bands and whorls, often with a striking parallel or regimental formation of the nuclei, a condition not infrequently seen in peripheral neurofibroma and even in leiomyoma. In some of these acoustic tumors this nuclear parade reminds one forcibly of the feather marking of a Barred Plymouth Rock fowl. The explanation of this is unknown. Mitotic figures are rarely seen.

There is also another quite constant feature, the zones of "reticular" tissue. These zones consist of cells with round and rather deeply staining nuclei and scant cytoplasm, lying in a much vacuolated matrix. There is often much free fat and lipoid in these foci and also hydrops of the cells. At first glance one notes a certain resemblance to glioma but neuroglia fibrillae are usually absent and the processes give, albeit often not entirely developed, characteristic staining reactions for fibroblasts or their products. Elastic fibres are not present except those derived from the blood vessels. The association with such foci of thrombosed or hyalinized blood vessels suggests that the "reticular" tissue is essentially the result of anemia of a portion of the tumor which formerly was probably of a dense fibrous nature like the rest of the growth. Isolated nerve fibers of acoustic nerve origin are rarely met with nor is there usually anything resembling a ganglion cell such as has been noted by Councilman in one of Cushing's cases. No corpora amylacea are present.

## Summary

1. Three cases of verified tumors of the cerebellopontile angle are presented. In one instance the tumors were bilateral and of practically the same size.

2. These tumors by virtue of their location give rise to a characteristic clinical picture.

3. The four tumors in the three cases reported are of almost identical histology and agree essentially with the descriptions of the tumors of the nervus acusticus as described by Cushing, Mallory, Councilman and Goodpasture.

4. They are fibromas with, however, slight deviation from the staining reactions of fibroblasts in tumors elsewhere. Their origin is apparently from the perineurium of the acoustic nerve and they have many points of resemblance to peripheral neurofibromata. A regimental or palisade arrangement of nuclei is practically always present in some degree. The so-called reticular tissue is probably the result of degeneration due to hemorrhage or thrombosis or other vascular injury.


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## DESCRIPTION OF PLATES XLIX–LII

### PLATE XLIX

FIG. 1. Case I, photograph of the base of the brain showing bilateral acoustic tumors in the cerebellopontile angles, producing marked pressure changes in pons and cerebellum.

### PLATE L

FIG. 2. Case II, photograph of the acoustic nerve tumor deeply embedded in the right lobe of the cerebellum.

FIG. 3. Case II, a vertical section through the cerebellum, showing the tumor to be well encapsulated but causing great pressure effects upon cerebellum, cerebellar peduncle and pons.

### PLATE LI

FIG. 4. Case I, a detail showing the parallel alignment of nuclei.

FIG. 5. Case II, a zone of "recticular" tissue (edematous and much vacuolated) with a thrombosed capillary and several capillaries with hyaline thickening of their walls.

### PLATE LII

FIG. 6. Case II, showing the fibrous nature of the tumor and in several places a parallel or regimental arrangement of nuclei. In the upper right-hand corner is a small focus of "reticular" tissue.

FIG. 7. Case III, parallel nuclear formation and an abundance of fibroglia.

NOTE. The photomicrographs were made from haematoxylin-eosin stained sections except Fig. 7, which was a phosphotungstic acid-haematin preparation.

# THE PATHOLOGY OF THE HYPOPHYSIS

## II. LYMPHOCYTIC INFILTRATION *

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In a study of more than 200 hypophyses in serial sections, 21 cases were encountered that showed areas of lymphocytic infiltration. The type of infiltration here reported is quite different from that which occurs about gummas and tubercles in the hypophysis, and there is little in the literature concerning it. The lesion was mentioned by one of us in a previous paper.[1] Pirone[2] observed lymphocytic infiltration in the hypophysis in hydrophobia; and M. Simmonds[3] and Berblinger[4] mentioned its occurrence in the vicinity of metastatic tumors in the posterior lobe. The 21 cases in our material have been studied with the hope of learning something of the nature and significance of the lesion. It has not been possible to investigate satisfactorily its relation to clinical symptoms because many of the patients from whom these hypophyses were obtained suffered sudden deaths and no anamnesis was obtainable.

It has been possible to divide these 21 cases into four groups as follows:

Group I. Those in which the area of lymphocytic infiltration was in immediate relation to one or more masses of colloid, either free in the tissues or inclosed in a space lined with epithelium.

Group II. Those in which the mass of lymphocytes was closely associated with one or more blood vessels and often resembled a potential lymph gland.

Group III. Those in which the lymphocytes diffusely infiltrated the tissues involved, without relation to blood vessels or colloid.

Group IV. Those in which the infiltration occurred as perivascular cylinders.

Border-line cases between Groups I and II occur. Without going into detail in individual cases, the following summary will suffice to present the essential features characteristic of each group.

* Received for publication February 24, 1925.

Each of the eleven cases in Group I showed one or more areas of lymphocytic infiltration in the middle lobe of the hypophysis. The location of these masses was rather constant. They lay very near the border line between the middle lobe and the neurohypophysis, frequently extending into the latter, and usually situated well laterally from the median plane. Furthermore, the infiltrating cells did not, in general, form a sharply circumscribed mass but gradually lost themselves in the surrounding tissues. In most of the cases the supporting tissue of the mass was the ordinary connective tissue which the cells had infiltrated. In two cases, however, the area suggested the follicle of a lymph node in appearance. The mass of infiltrating cells in almost all cases was quite near a small blood vessel. In all except one case it was intimately associated with colloid material, either lying free in tissue spaces, or inclosed in small "cysts." These cysts were more or less completely lined with low columnar or cuboidal epithelium and contained colloid that was vacuolated and strewn with desquamated epithelial cells. In one case, the desquamated cells had fused into a sort of syncytial mass containing a dozen or more nuclei. In two cases, plasma cells were present, either in the edge of the area of infiltration or nearby in the surrounding tissues. However, collections of colloid in the hypophysis are not always associated with lymphocytic infiltration.

The ages of the patients of Group I ranged from 20 to 50 years. All except two were 35 or more years old. There were only two women in this group. The pathologic changes found in other organs of the body varied greatly. One patient died from mitral stenosis, another from tumor of the brain, another from hemorrhagic encephalitis, the fourth from rheumatism and an ulcerative colitis of of undetermined origin, and the fifth from chronic meningitis three months after fracture of the skull. The remainder of the patients of this group died as a result of trauma. Microscopic examination of sections of different organs for areas of lymphoid infiltration was without result except in the case of the liver from the patient with ulcerative colitis.

Group II includes six cases. In these the supporting tissue in the areas of lymphocytic infiltration was typical reticulum. In all six cases, with possibly one exception, cells were present which resembled those that make up the germinal centers of a lymph node. These cells were not massed together as in a typical germinal center,

but were diffusely scattered among the lymphocytes in the central portion of the area. The infiltrations in this group were rather sharply circumscribed but did not possess a capsule. No plasma cells were found in or near the lesion in any case. The mass of lymphocytes in typical instances was not associated with colloid, either free or inclosed in epithelium-lined spaces. They did, however, have a rather constant and characteristic relation to blood vessels.

The posterior lobe of the hypophysis extends downward and forward into a cup-like depression in the anterior and middle lobes. In the median plane, its tip is separated from the middle lobe at the bottom of the depression by a mass of connective tissue in which is a plexus of blood vessels numbering from 5 to 20 in different sections. The infiltrating mass of lymphocytes in this group usually lies within this plexus between adjacent blood vessels. There is no tendency for the lymphocytes to surround one or more of the vessels, hence this lesion is distinctly different from perivascular infiltration.

This group contains four men and two women. The youngest patient was 12 years old, the ages of the others ranged from 35 to 55 years. One died of generalized miliary tuberculosis with tuberculous meningitis; another with chronic ulcerative tuberculosis of the lungs; one from chronic diffuse nephritis; a fourth from cerebral hemorrhage. All of these patients, except the 12-year-old girl, had varying degrees of arteriosclerosis.

In Group III (with two cases), the lymphocytic infiltration occurred in the anterior lobe. In one instance it involved the middle lobe and extended forward into the anterior lobe; in the other, it was limited to one lateral half of the glandular portion of this organ, which showed also a chronic hypophysitis. The infiltration in each case was diffuse, more so in one case than in the other. No plasma cells were found. One of these patients was a young woman, 20 years of age, who died of chronic alcoholism and exposure. The other, with the chronic hypophysitis, was a middle-aged man who died of lobar pneumonia.

In each of the cases of Group IV the infiltration was perivascular and located in the posterior lobe, usually near the capsule. The first of these patients suffered from an acute streptococcus meningitis, but the cylinders of perivascular infiltration were composed of lymphocytes. The second patient was an elderly man with marked chronic

myocarditis and arteriosclerosis of the coronary arteries, who had, two weeks before death, suffered an injury to the head which caused a slight hemorrhage in the pia and superficial part of the brain. The infiltrating cells in this case were different from those in any other case of this series, in that they resembled transitional or endothelial leucocytes.

## Discussion

The nature and significance of the lesion in these 21 cases is perhaps different for each of the four groups. While lymphocytic infiltration in the hypophysis of the type here described does not appear to have been previously reported; it has been described in the thyroid and adrenals. M. Simmonds [6] found areas of lymphoid infiltration in 20 per cent of normal thyroids and in 80 per cent of thyroids in exophthalmic goiter. In this gland the lesion may appear in any degree from a small accumulation of lymphocytes to large areas with germinal centers and resembling in all essential respects, a follicle of a lymph gland. MacCallum [7] thought that lymphoid infiltration of the thyroid in toxic goiters was due to the action of toxic or injurious substances. M. Simmonds [8] believed that the accumulations of lymphocytes in the thyroid represent a reaction caused by the effects of an abnormal secretion. Kocher [9] observed areas of lymphocytic infiltration in both exophthalmic and adenomatous goiters, both in the adenomas and in the adjacent tissues. They were not, however, characteristic of toxic goiters because they were occasionally found in normal thyroids and in simple strumas. In most cases the lymphocytic infiltration was not related to blood vessels; in a smaller number, it was perivascular. The accumulations of lymphocytes were most frequent in those sections in which the acini showed absence of colloid and multiple layers and desquamation of the epithelium, and in areas of atrophy of the thyroid tissue. Kocher considered lymphocytic infiltration as an expression of chemical changes in the thyroid. V. Gierke [10] thought that lymphoid infiltration in exophthalmic goiters was associated with status thymico-lymphaticus. With this view both Simmonds [8] and Kocher [9] disagree.

Paunz [11] and others have described round-cell infiltration in the adrenals. Paunz thought that this condition was a reaction to a chronic infection or to a chronic intoxication which might be of

endogenous origin. This would partially explain the more frequent occurrence of this lesion in persons of middle age or older.

In as much as lymphocytic infiltration in the hypophysis occurs chiefly in or near that part of the gland which contains colloid, it would seem that this lesion in the hypophysis might be somewhat similar in nature to the similar condition in the thyroid. In Group I of our series of cases the similarity is all the more apparent because in this group the areas of lymphoid infiltration were definitely related to the presence of colloid. When the colloid was free in the tissue spaces, as in Case 1, it might stimulate the accumulation of lymphocytes just as any other foreign body might do. In those cases in which the colloid was inside an epithelium-lined cavity, it appears to be abnormal because: (1) it is vacuolated; and (2) contains many desquamated epithelial cells, as in the thyroid in many cases of toxic goiter; and (3) the epithelial lining of the "cyst" which contains the colloid is often abnormal in arrangement and in the number of layers of its lining cells. Whether, in these cases, the colloid is actually changed in quality it is not possible to determine from the data at hand. This possibility is suggested, however, by the fact that not all colloid-filled spaces are associated with round-cell infiltration.

None of the patients in our series showed any evidence of status thymico-lymphaticus. In only one was the thymus still present, and this was a young man 20 years of age. It is our opinion, therefore, that the lymphocytic infiltration in Group I is due to some local cause, possibly some change in the character of the secretion of the middle lobe. The presence of occasional plasma cells lends support to this view.

There is nothing about the accumulations of lymphocytes in the cases of Group II, on the other hand, that indicates a local cause. They occur, in their characteristic form, in a plexus of blood vessels, and are not directly related to the presence of colloid. The larger ones resemble an imperfect follicle of a lymph node: (1) in their rather sharply marked boundaries; (2) in the arrangement of the component lymphocytes; (3) in the reticular type of supporting tissue; and (4) in the presence of large cells with vesicular nuclei similar to those in the germinal centers of lymph glands. In other words, the areas of lymphoid infiltration in this Group are essentially like the lymphocytic accumulations in the lungs described by Arnold,[12] and in the

liver by Ribbert,[5] Marcuse,[13] and others. None of the patients in this group showed any indications of status thymico-lymphaticus. Three of them had tuberculosis, another, chronic nephritis; and two had marked arteriosclerosis. Hence it is possible that in this group of cases the occurrence of masses of lymphocytes in the vascular plexus of the hypophysis may be related to chronic infection or to chronic intoxication, as Paunz[11] thought was the case in the adrenals.

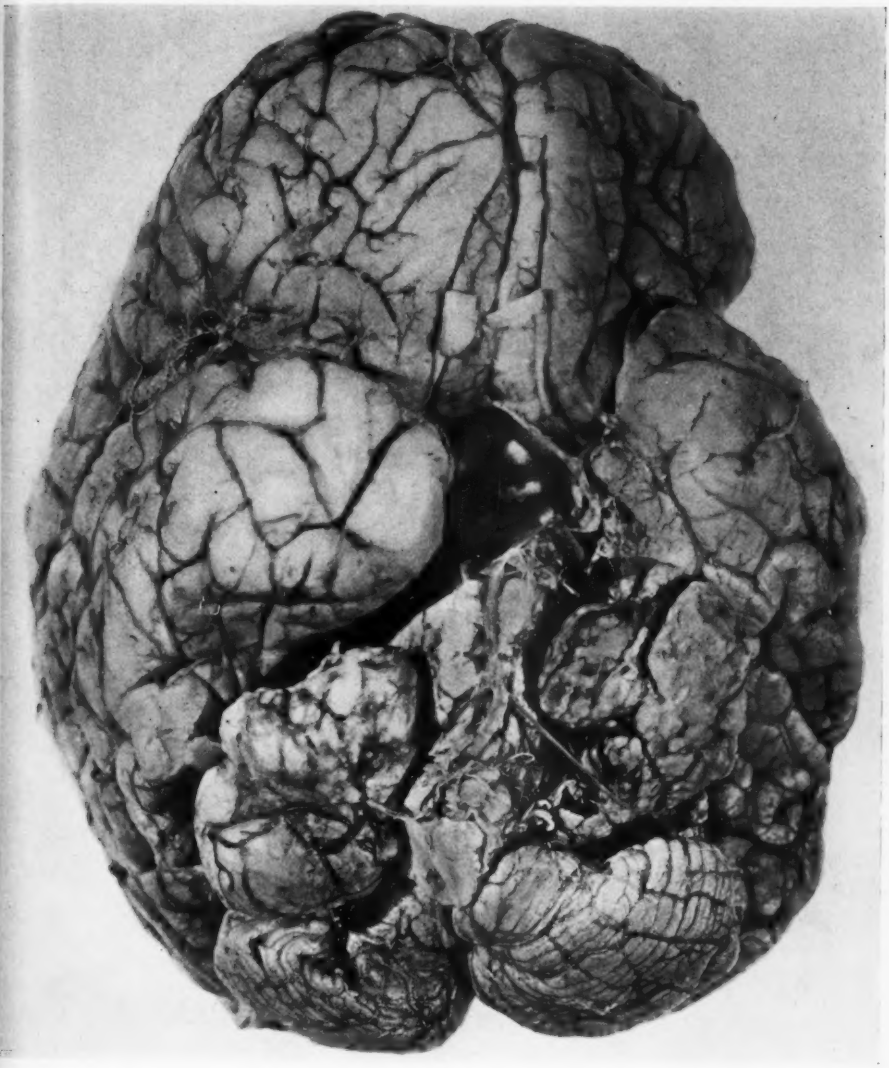
The presence of this type of lesion in the hypophysis is significant in another respect. Such accumulations of lymphocytes in other organs, as pointed out by Ribbert [5] in the case of the liver, are intimately associated with lymphatics. The presence of these areas of lymphocytic infiltration is suggestive evidence, therefore, contrary to the opinion of Thaon,[14] that this gland does possess lymph vessels.

Of the two cases in Group III, the lesion is easily explained as a part of a chronic inflammatory process in the anterior lobe of the hypophysis. The first case of this group, however, is the only one of its kind in this series, and we are unable to offer any plausible explanation of its presence.

In Group IV the infiltration is perivascular. In the first case the infiltrating cells were lymphocytes and the lesion was evidently an extension along blood vessels of an inflammatory process in the meninges. Simmonds [15] mentions involvement of the hypophysis by perivascular extension in cases of tuberculous meningitis. The lesion in the second case was characterized by an accumulation of transitional or endothelial leucocytes about a blood vessel. It is not possible at present to suggest any explanation as to its cause or nature.

While these 21 cases fall rather easily into the four groups outlined above, no symptoms or physical findings were common to a sufficient number to indicate the existence of a clinical entity corresponding to the histologic lesion. The study of the patient and the post-mortem examination of the body were not made with this distinct object in view, however.

It is perhaps justifiable to consider this round-cell infiltration of the hypophysis as a definitely pathologic process, in the nature of a reaction to some sort of stimulus. It will be remembered that Simmonds [8] found lymphocytic accumulations in 20 per cent of thyroids that were apparently normal. We have found this lesion in approximately 10 per cent of all of the hypophyses examined. Too little is



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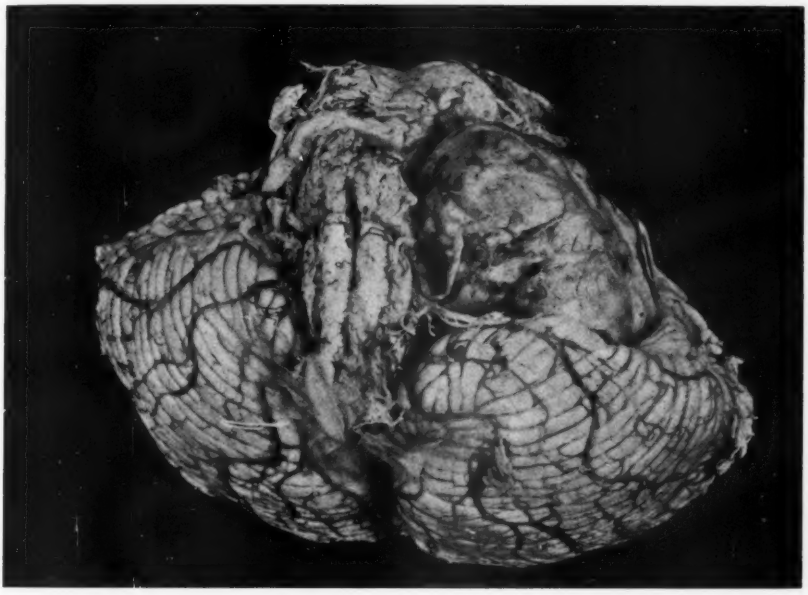
Jacobson Fibroma of the Acoustic Nerve



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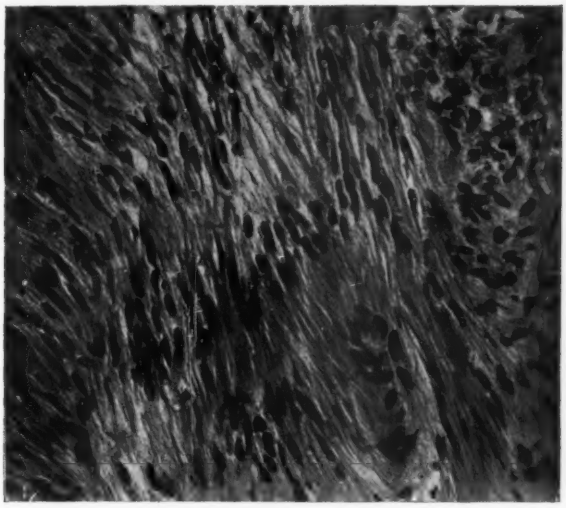
Jacobson Fibroma of the Acoustic Nerve



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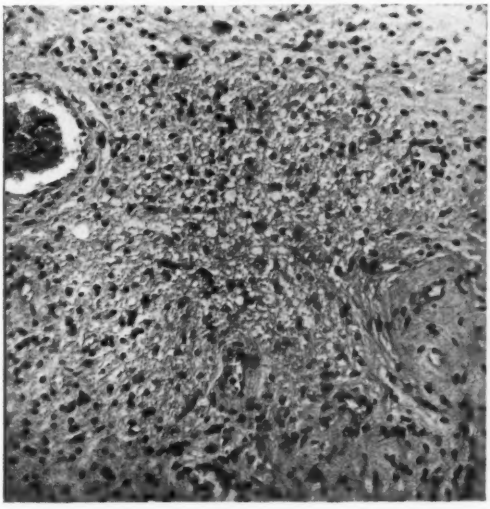

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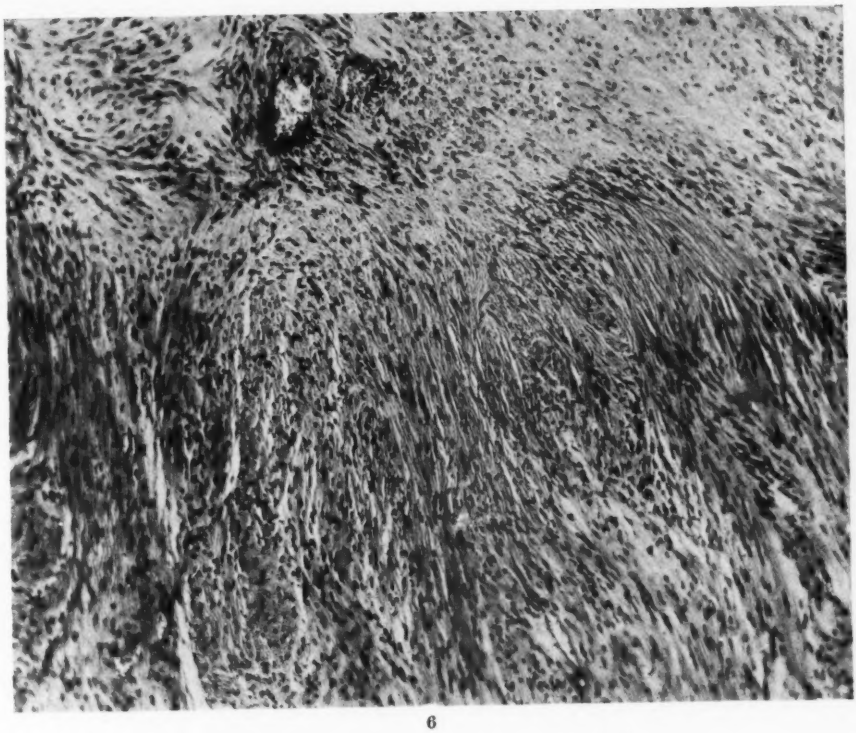

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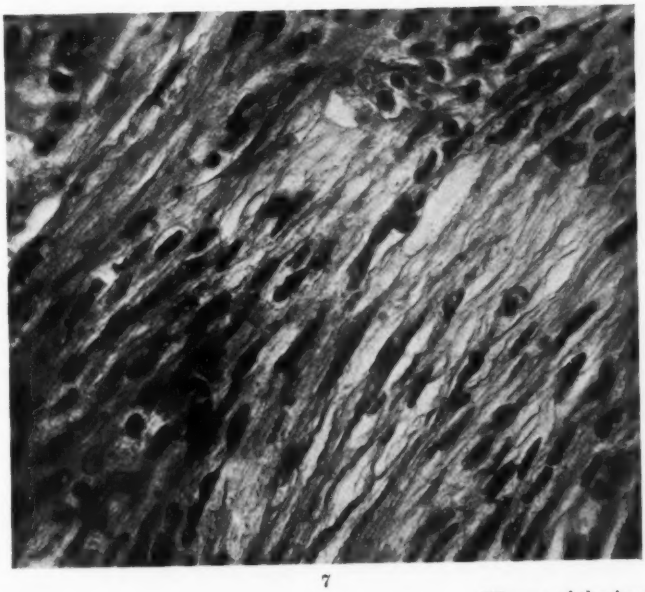

7

Jacobson

Fibroma of the Acoustic Nerve

known of the pathology of this gland to state with certainty whether many of those examined were normal or abnormal. However, we are inclined to consider the lesion in Groups I, III, and IV as a reaction to some local stimulus or irritant, and in Group II to some extra-hypophyseal stimulus. To what extent this process or the condition which caused it interferes with the function of the hypophysis cannot now be stated.

## Summary

1. Twenty-one cases of lymphocytic infiltration of the hypophysis, a lesion that does not appear to have been previously described in detail, are reported.

2. The process occurs most frequently in the middle lobe, less commonly in the anterior and posterior lobes.

3. It has been found in about 10 per cent of all hypophyses examined, and has been compared with the similar condition sometimes found in the thyroid and adrenals.

4. In the hypophysis the lesion appears in four distinct forms, three of which are believed to be the result of reaction to a local stimulus, the other to be associated with chronic infection of some distant organ or with some chronic intoxication.

5. The data available are insufficient to justify an opinion as to the effect of this change on the function of the hypophysis.

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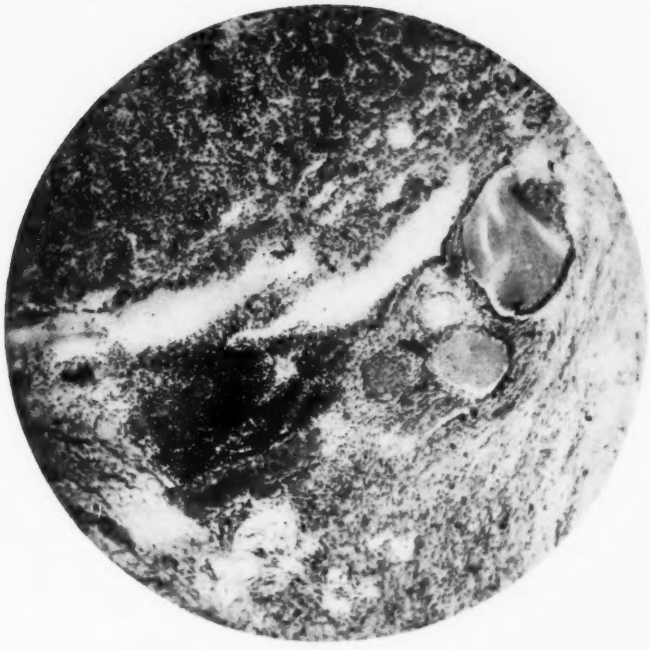
## DESCRIPTION OF PLATE LIII

FIG. 1. Area of round-cell infiltration associated with colloid. Immediately below the area of infiltration is an acinus in which the epithelium is desquamated. X 70.

FIG. 2. Round-cell infiltration adjacent to blood vessels (at the left) and to abnormal acini (at the right). X 130.



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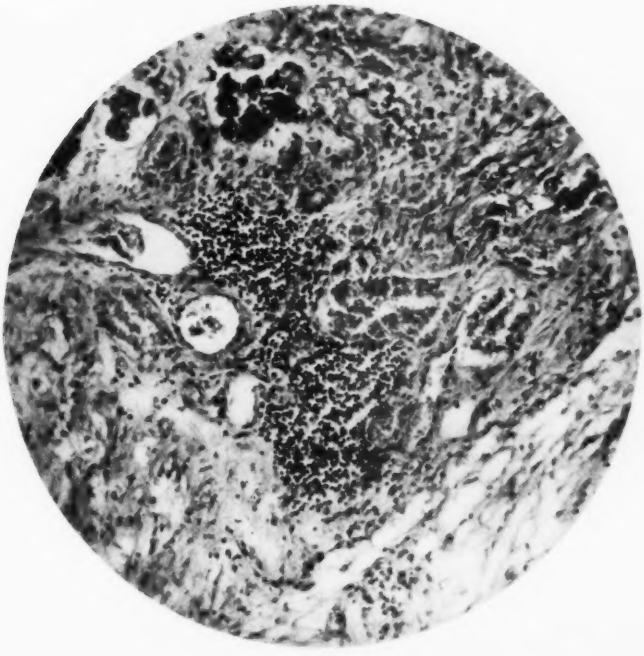

2

# AN ADENOCARCINOMA RESEMBLING THE THYROID GLAND *

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Neoplastic formations in the lower animals in tissues which resemble the pattern normal to the thyroid gland are of infrequent occurrence. The veterinary pathologist occasionally sees heterotopic manifestations of various organs but for the most part the tissue elements comply with the requirements of the architectural design characteristic of the tissue or organ duplicated and seldom exhibit a progressiveness of such nature as to qualify as a tumor.

In the following we have a structure that is unusual enough in its design and location to warrant description, although the factors bearing upon its etiological aspects will probably remain a matter of conjecture.

*Case Report.* In October 1923, Dr. G. G. Feldman, veterinary inspector in the United States Department of Agriculture, Bureau of Animal Industry, stationed at Spokane, Washington, forwarded to our laboratory a tumorous mass with the following history: A five-year-old grade cow was presented for slaughter. A spherical formation about six inches in diameter was observed attached under the skin over the muscles of the neck lateral and just posterior to the first cervical vertebra. After slaughter the mass was removed and placed in formalin fixative for future microscopic diagnosis and study.

*Macroscopic appearance of tumor.* The tumor was spherical in shape about six inches in diameter, fairly firm in consistency and of a flesh color which gradually faded to a whitish gray. Over the unattached surface under the skin a capsule was present. The surface was smooth without ulceration and covered with hair. It was attached rather broadly to the underlying structures by the connective tissue and skin. The mass contained a considerable amount of blood but no pigments or cysts were observed.

* Received for publication February 27, 1925.

*Pathological histology.* Sections were prepared from various portions of the submitted material and stained with hematoxylin and eosin.

The low-power view presented an atypical structure containing a number of irregularly shaped alveoli lined with cuboidal epithelium. For the most part of the cells lining the alveoli were in a single layer, although many alveoli showed an excess of these cells usually at one side, as though infiltrating into the adjacent stroma (see Fig. 1). Most of these alveoli were filled with a clear homogeneous substance that stained a bright pink with eosin, and which gave the characteristic orange-yellow reaction of epithelial hyalin, with Van Gieson's stain, although some of the material within the alveoli gave a light rose red reaction by the same method. In the areas between the alveoli the connective tissue was scant. Most of the space was occupied by what appeared to be epithelial cells morphologically similar to the cells lining the alveolar spaces. These cells were closely packed in small nestlike compartments, which arrangement was very apparent under the high power (see Fig. 2). Some fields contained large irregularly shaped areas, consisting of a hyaline-like substance which appeared vacuolated, and which stained a lavender color by Van Gieson's method. The nestlike groups of epithelial cells were few in these areas, and the number of cells making up the individual groups was a great deal less than in the other parts of the field and many showed changes suggestive of atrophy. Around many of the epithelial nests, and in the lumina of most of the alveoli were many vacuoles of variable size and shape (see Fig. 1). Red blood corpuscles were abundantly distributed in capillaries throughout the scant connective tissue of the stroma, and large blood vessels with well-defined walls were numerous. A few of the larger vessels showed thrombi formation apparently of some standing.

Viewed under high power the individual epithelial cells were spherical and oval in shape, with considerable variation as to size and staining capacity of the nuclei. Some, mostly of the small variety, staining with deep intensity so that chromatin granules were not discernible while others stained lightly except for the chromatin material which appeared as minute granules. The nucleus occupied most of the cell, the cytoplasm being so small in amount as to escape detection in most instances. Actual mitosis was not prevalent, al-

though there was enough evidence of this to indicate some aggressiveness on the part of the tumor cells (see Fig. 3).

*Diagnosis.* The epithelial character of the cells arranged in alveolar and nestlike formations, together with evidence of mitosis and infiltration would stamp this as an adenocarcinoma.

There is sufficient evidence, I think, to feel reasonably sure that the type cell in this formation had a common origin with the epithelial cells making up the parenchyma of the normal thyroid gland. The tendency toward alveolar arrangement and the presence of colloid material makes this obvious, even though the attempt at alveolar formation was not successful throughout. However when one considers the location of the tumor it is somewhat difficult to give an acceptable explanation of its position that is in keeping with current embryological teaching although its heterotopic origin is possible from some prenatal mishap, involving one of the lateral thyroids. From the standpoint of a metastatic tumor from the thyroid gland the task of explaining its presence would not be so difficult if it were possible to examine the gland, as it might present some interesting features bearing upon the possible origin of this tumor. Any change in the thyroid in this animal apparently was not obvious, otherwise the gland would have been removed at the time of slaughter.

While the bulk of the structure was decidedly infiltrative in character, yet the scarcity of mitotic figures together with the age of the animal and the apparent lack of metastatic foci would indicate a tumor of slight progressiveness.

Some of the microscopic features are difficult of interpretation, especially the hyaline-like areas which gave the atypical reactions to Van Gieson's stain. It is my opinion that the substance making up these areas and the hyaline contents of the alveoli had a common origin, and simply present different stages of the same material. Here we are confronted with the usual obstacles one encounters when attempting adequately to describe and interpret a hyaline change.

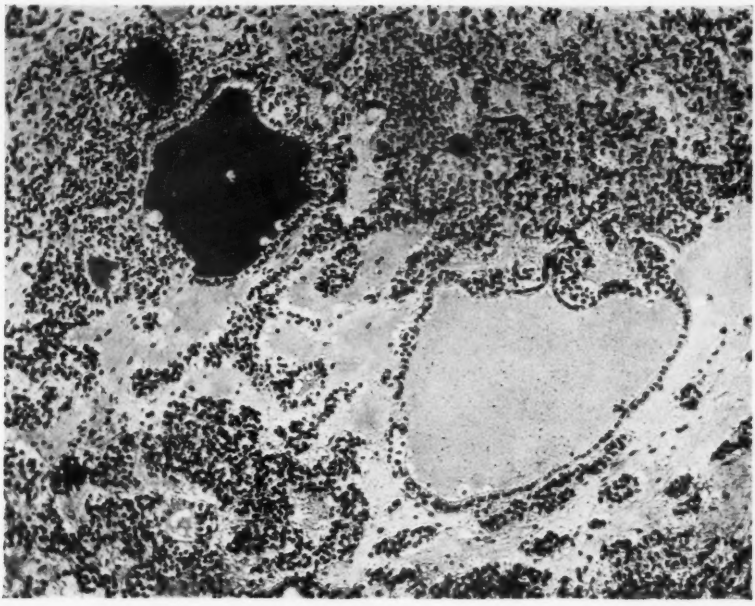
The vacuoles are possibly the spaces vacated by the removal of fat droplets since the tissue had been preserved in alcohol for some time. For this reason there was no opportunity of preparing sections for a specific fat stain.

## DESCRIPTION OF PLATE LIV

FIG. 1. Adenocarcinoma resembling the thyroid gland. Low-power photomicrograph, showing irregular hyaline-like areas, alveoli, vacuoles, etc.

FIG. 2. Adenocarcinoma resembling the thyroid gland. High-power photomicrograph showing nestlike formations of tumor cells.

FIG. 3. Adenocarcinoma resembling the thyroid gland. High-power photomicrograph showing one cell undergoing mitotic division.



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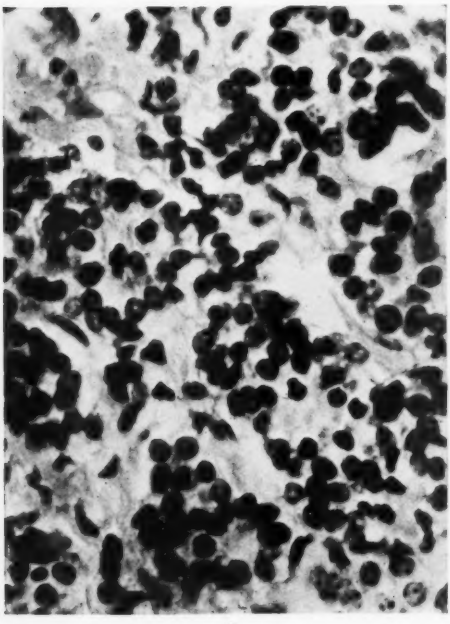

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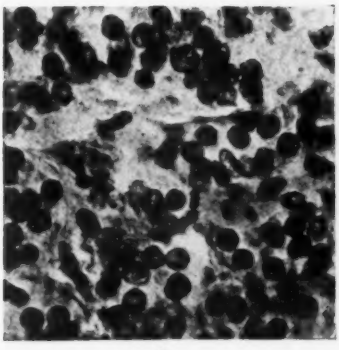

3

Feldman

Adenocarcinoma Resembling Thyroid Gland

# TISSUE CHANGES AFTER EXPERIMENTAL DEEP ROENTGEN IRRADIATION *

ANATOLE KOLODNY, PH.D., M.D.
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It is a well-recognized fact that the influence of Roentgen irradiation upon the organism as a whole is just as important as its influence upon the pathological condition itself and may be more so. That fatal intoxication may be a sequel to intensive Roentgen therapy is a fact which is known from the altogether too rare publications of such misfortunes. Patients treated intensively with Roentgen rays very frequently react with general malaise, prostration, vomiting, diarrhea, loss of appetite, weak pulse and headache, as may be ascertained by anybody who has had the opportunity to observe even a limited number of such patients.

In the opinion of Miescher [1] the clinical incidence of radiation toxemia is about forty per cent of all cases treated, this condition being most marked when the radiation was over the epigastric area. Groedel and Lossen [2] met with radiation toxemia in twenty-five per cent of all cases when the head and neck were exposed, in thirty-three per cent in irradiation of the thorax and in fifty per cent in irradiation of the abdomen. This constitutional reaction does occur, though varying in degree, not only in deep Roentgen therapy but also in irradiations with the long-waved soft rays, a fact which has not been sufficiently stressed in the literature. Twenty years ago Seldin [3] tried to explain the cause of "Roentgenkater" by experiments on guinea pigs. A line of investigators followed him in an attempt to solve this difficult question. Some brilliant work was done recently by American and British investigators who contributed much to the pathological physiology of irradiation intoxication. However, this question is still far from a satisfactory solution.

What is the cause of irradiation toxemia? To discuss the literature of this question would mean that one would become lost in a maze of opinions. This may be illustrated by the mention of just a few of the multitude of theories and hypotheses: Gas poisoning

* Received for publication January 30, 1925.

through poor ventilation of the Roentgen room, induced high voltage of the patient's body, toxic nephritis with acute uremia, retention in the body of toxic products of nucleo-protein decomposition, anaphylaxis, psychical sickness. Lange[4] speaks of acidosis developed in the irradiated body as the cause of intoxication and advises prophylactic treatment with alkalies, while Myers[5] speaks of alkalosis in which alkali therapy would greatly aggravate the condition. As may be seen this question of the cause of irradiation toxemia is still a field of speculation where hypotheses greatly overwhelm proved facts. There are, however, features in the pathology of Roentgen intoxication which are of interest even if they alone are insufficient to explain the condition. These features are the morbid anatomical changes.

In 1921 Wetzel[6] in Europe and in 1924 Case and Warthin[7] in this country called attention to certain changes in the liver observed in patients who were treated with deep Roentgen therapy and died afterwards. In the pathological study of patients who have died after therapeutic irradiation the pathology of the condition for which radiation was done has to be considered. This fact makes the very limited human material available for pathological study of questionable importance. It is evident, therefore, that the pathology of Roentgen irradiation may be best studied by experimentation on animals.

Most experimental work in this line has been done since the invention of the Coolidge tube. From the published reports it would seem that most authors did not pay due attention to the question of the amount of X-ray energy given the experimental animal. The data usually given in the reports: the voltage, skin target distance, time of treatment, amperage and filter, are not sufficient for an exact measure of the amount of X-ray energy given. Insufficient attention has been paid to the readings of the electrostatic indicator, which vary with the tube used and vary also in the same tube at different times. There can also be no question that if a guinea pig is exposed to the same amount of X-ray energy as a dog, or a dog to the same amount as a human being the morbid changes produced will not be characteristic of the changes produced in patients by Roentgen therapy. Inattention to these facts may explain some of the discrepancies in the opinions of the investigators as to the results of the experiments.

In this work an attempt has been made to study the morbid changes in the liver, kidneys, spleen, and intestinal tract in experimental irradiation of rabbits. Two series of experiments were conducted. In one the rabbits were exposed to an X-ray energy, in proportion to their average weight, about twice as high as the energy to which patients are exposed in the usual intensive deep Roentgen therapy. The amount of X-ray energy and the dose given these rabbits can be calculated from the following data: 5 milliamper tube current, 200.000 volts (peak), filter 0.53 mm. Cu., skin target distance 70 cm., 1950 "e" units of X-ray energy per sq. cm. exposed, the time of exposure two hours. The whole body was exposed. In the second series the epigastric region of the rabbits was exposed; the time of exposure one hour. All the other conditions in reference to the X-ray energy and the dose given were the same as in the first series. Under these conditions the rabbits of the second series were given an irradiation, in proportion to their average weight, equal in intensity to that which is usually given to patients in deep Roentgen therapy. All rabbits died from the third to the ninth day after exposure. There was no appreciable difference in the changes in the organs studied between the two series.

The gross anatomical findings in the organs other than those described below were of no interest. The liver, the kidneys, the spleen and the intestinal tract were studied in gross and microscopically. They showed the following changes.

*Liver.* There were no appreciable gross changes in the liver except for scattered pinpoint- and pinhead-sized areas which could be perceived by means of a middle-power magnifying lens. These areas were distinctly different from the surrounding liver tissue. Their gross characteristic, however, did not permit any conclusions as to their nature. Microscopically one sees in the liver passive congestion which is especially prominent during the first few days after irradiation. Beneath the capsule of the liver there is marked dissociation of the liver cells with swelling of the cytoplasm and weak staining of the nuclei. The cytoplasm has a granular appearance. Deeper in the liver parenchyma are found scattered numerous small necrotic foci. Here may still be found liver rods, composed of cellular elements, which have undergone necrotic changes. These necrotic areas are surrounded at their periphery by liver cells still with staining nuclei but with broken-down cytoplasm, showing marked hy-

dropic changes. In the immediate vicinity of the necrotic foci congestion is very prominent. Here is seen some lymphocytic infiltration, especially in the older cases. Further to the periphery the liver cells are pyknotic, the nuclei stain deeper but seem of smaller size without the characteristic vesicular appearance. The Kupffer cells throughout the liver contain granular inclusions, consisting of chromatin and hemosiderin. The blood vessels in the necrotic areas are thrombosed; in some of them the organization of the thrombi is well advanced.

The last to undergo degeneration seem to be the bile ducts, so that in distinctly necrotic areas more or less normal bile ducts are still found. However, in the central portions of the necrotic foci the bile ducts are necrotic, traces of the columnar lining epithelium and accumulations of bile pigment scattered in the necrotic tissue being the only signs of former sites of bile ducts. On the whole a hyperplasia of the bile ducts is seen as may be judged from the character of their lining epithelium as well as from the rapidity with which they regenerate in the necrotic foci.

At a later date (five to eight days after exposure) one finds an interesting picture of repair in the injured liver. In the central portions of the necrotic foci are still present masses of cellular débris, which have not been as yet removed by autolysis, absorption and phagocytosis. The periphery of the necrotic areas begins to show all signs of repair. Connective tissue has spread from the periportal spaces far into the necrotic foci. Young fibroblasts with fusiform and vesicular nuclei have invaded this area. In the peripheral portions, where repair began at a somewhat earlier date, may be found groups of liver cells invading the repaired area. These liver cells do not show the degenerative changes found in the old liver tissue surrounding the necrotic foci; they seem to be new-formed elements. In the area of repair may be seen old bile ducts which did not succumb at the time when the liver cells became necrotic; but also numerous new-formed bile ducts are found. Some of the latter show small lumina, lined with more or less cuboidal epithelium. The majority are, however, in the very young stage when no lumina are seen as yet. They are formed by long groups of cells with relatively large nuclei and small amount of cytoplasm. On a cross section they appear as giant cells with nuclei placed at or about the periphery, with the center (lumen) taken up by the syncytial cytoplasm which

does not show as yet clear cell borders between the individual cellular elements. Special staining shows the connective tissue wall surrounding these young ducts. Such "giant cells" could never be found in lumina of larger bile ducts. Considerable thought and careful examination was given to this question, because of a recent paper by Case and Warthin [7] in which the authors speak of giant cells obliterating the bile ducts in the livers of patients who died after intensive deep Roentgen therapy for abdominal malignancy.

*Kidneys.* Several theories attempting to explain the irradiation toxemia are based upon the temporary depression of the function of the kidneys. The recent hypothesis of Anderson and Kohlmann [8] that Roentgen sickness is due to an acute uremia is the most well-developed of these theories. However, the question of the changes in the kidneys following intensive irradiation is still an open one. In these experiments the kidneys showed at all times after irradiation an extensive focal parenchymatous nephritis. Congestion and cloudy swelling of the cortex were the main characteristic changes in these foci. The desquamated lining cells of the tubuli frequently formed granular casts. The glomerular tufts showed the same degenerative changes to some slighter degree. Free blood was found frequently within Bowman's capsule. The medullary portion of the kidneys did not seem to be affected by the process.

*Spleen.* The spleen is markedly changed grossly. The decrease in size is astonishing. The capsule is redundant and laid in folds. As a matter of fact in two cases it was difficult to find the spleen at autopsy. In each rabbit only a small piece of dark gray tissue was present at the splenic site.

The changes are even more marked on microscopic examination. Only very small remnants of the Malpighian bodies are left. The main bulk of the remaining splenic tissue is made up of the splenic pulp. Moreover there is a conspicuous scarcity of lymphoid cells in the pulp. Very few lymphoid cells can be found in the remaining small Malpighian corpuscles. The leading elements of the small corpuscles now are the long spindle-shaped cells with bulging nuclei. In contrast to the liver and kidneys the spleen is very anemic. The central arteries and cavernous veins seem to be collapsed, especially the latter. At the same time there are seen in the reticular meshes numerous broken-down red blood cells.

The large ameboid phagocytic cells of the splenic pulp contain abundant granular inclusions consisting in the main part of hemosiderin. Along with hemosiderin these inclusions contain cellular débris of disintegrated lymphocytes. As may be seen here, as in the liver, the reticulo-endothelial apparatus participates in the phagocytosis of the remnants of the disintegrated cellular elements and especially of the end products of the disintegrated red blood cells.

*Intestinal tract.* The changes in the intestinal tract after intensive irradiation have long been known from experimental as well as from clinical study. The intestinal tract permits the best systematic study of its anatomical changes after irradiation. One finds there different degrees of involvement from the most inconspicuous desquamation of the epithelium lining the tips of the glands of the intestinal mucosa to the most serious deep ulcerations of the intestinal wall with impending perforation of the gut. In twelve hours after epigastric irradiation may already be found a desquamation of the mucosa epithelium, no hemorrhage or cellular infiltration being present as yet. Soon changes in the blood vessels appear. The endothelium lining them swells, becomes granular and desquamates. Thrombosis of the vessel follows. The extensive thrombosis leads to necrosis, to ulceration of the intestinal mucosa and submucosa. A hemorrhage in the immediate vicinity of the necrotic area leads to a picture which can be well seen grossly as a dark red ulcer of the mucosa. Necrosis spreads deeper into the wall and in one case was found a perforation of the intestinal wall with a subsequent general peritonitis. A marked lymphocytic infiltration was found to be constantly present in the pathological picture. However, the lymphocytic infiltration is of a far less degree than one would expect in such a process as an ulceration of the intestinal mucosa. In contrast to the liver one does not see any signs of repair in the intestinal mucosa. In all cases the ulcers progressed until the death of the animal took place. In one of the rabbits ulceration occurred also in the vermiform appendix of the cecum, thus producing an acute gangrenous appendicitis which led to perforative peritonitis and death.

## Discussion

Such are the morbid changes which took place in the organs of the experimental animals. An analysis of these results shows certain features which are common to all of the organs studied. One of these

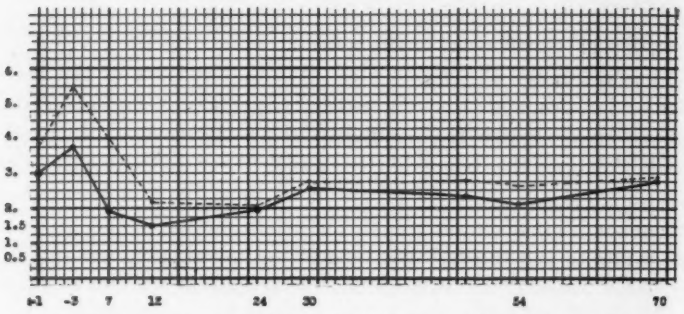
is the changes in the blood vessel walls, changes which lead to extensive thrombosis. The destruction of the erythrocytes is another characteristic feature of the changes induced by intensive Roentgen irradiation. But the most conspicuous of these features is the rarity and scarcity of lymphocytic infiltration accompanying the inflammatory and necrotic changes of the tissues, and the enormous destruction of lymphocytes in the spleen.

The destruction of lymphocytes, the disappearance of the Malpighian follicles, and the overgrowth of the reticular elements of the splenic pulp suggest the possibility of an influence of Roentgen irradiation upon the coagulability of blood. The two leading theories regarding coagulation of blood are based upon the hypothesis that the spleen plays a specific rôle in the determination of this process. According to one the reticular cells of the splenic pulp are specific for blood coagulation. Stimulation or increase in number of these elements will therefore lead to an increase in coagulability of the blood. The other theory, supported by the majority of investigators, claims that a destruction of the splenic follicles leads to a liberation of thrombokinetic or thromboplastic substances. The changes in the spleen of the rabbits are of such a nature that accepting either one of these two theories an increase in coagulability of the blood after irradiation could be expected.

To study the influence of irradiation upon coagulability of the blood two series of experiments on rabbits were conducted. The epigastric region in normal rabbits was exposed to X-rays. The dose of X-ray given was, in proportion to the average weight of the rabbits, equal to about one fifth of an intensive therapeutic dose for a human being. The coagulability of blood was studied after the method of Brodde-Rossell and by the capillary method. The curve given in text figure 1 illustrates the findings. It may be seen that there is a definite increase in coagulability of the blood (a decrease of time in which coagulation takes place) after irradiation. The highest coagulability was observed at about twelve hours after the exposure. Experiments on rabbits in which various other regions were exposed gave similar results, though the increase in coagulability of the blood was less marked.

A further study of the literature showed that in 1920 Stephen [9] discovered that irradiation by Roentgen rays increases the coagulability of blood. He then suggested the use of irradiation in surgery

when an increase in coagulability is necessary for a surgical interference. This suggestion has not gained ground, apparently because of the fact that the influence of irradiation upon coagulability was not understood biologically. Though the question of the influence




TEXT FIG. 1. Curve illustrating the influence of Roentgen irradiation on the coagulability of blood. The continuous line is the result of the Brodde-Rossell method. The broken line of the capillary method.

of irradiation upon coagulation of blood has not been wholly answered by the described results of these experiments, it would seem, however, that the pathological changes in the spleen after irradiation suggest the biological explanation of this influence.

## CONCLUSIONS

Experimental intensive deep Roentgen irradiation produces definite anatomical changes in the liver, the kidneys, the spleen and the intestines, which play a certain rôle in the etiology of radiation toxemia.

The main features of these histopathologic changes are:

1. A destruction of lymphocytes, which is especially prominent in the spleen.

2. A disintegration of erythrocytes in all the organs studied with abundant hemosiderin formation.

3. Degenerative vascular changes with subsequent thrombosis of the vessels.

The destruction of lymphocytes and the disappearance of Malpighian follicles of the spleen with the overgrowth of the reticular elements of the splenic pulp are the biological basis for the increase of the coagulability of blood after irradiation.

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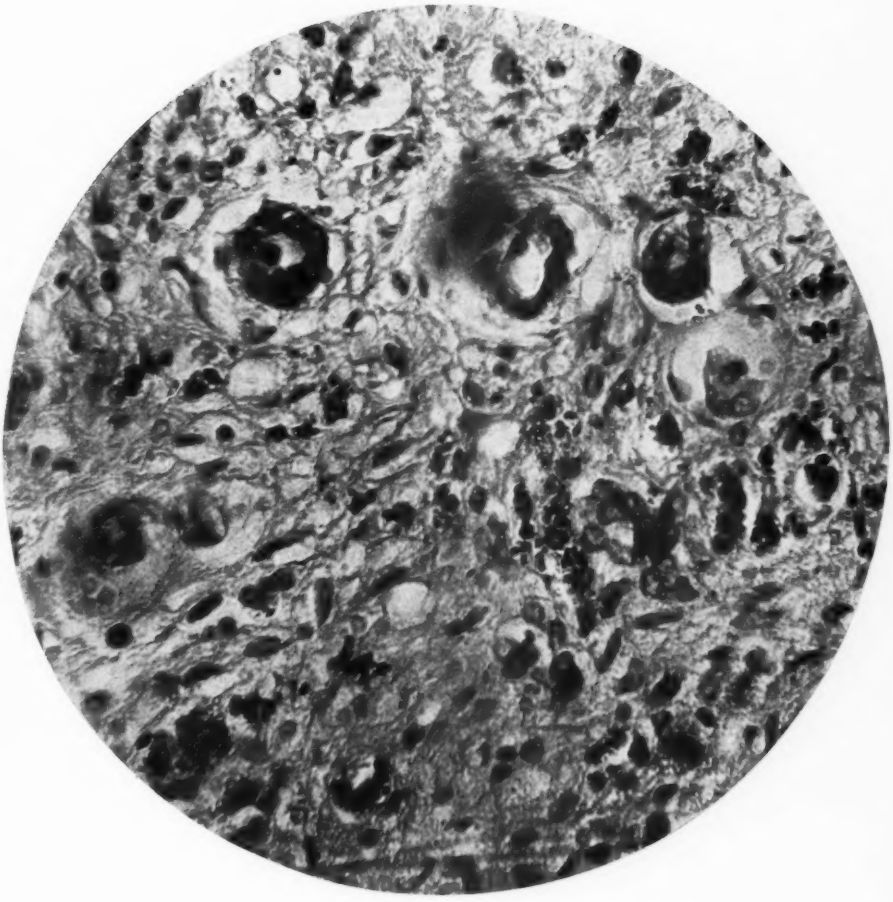
## DESCRIPTION OF PLATE LV

FIG. 1. Regeneration of bile ducts in a necrotic focus of the liver where repair is well advanced. The youngest of the bile ducts do not show as yet any lumina, and on cross section they simulate giant cells.



Kolodny Tissue Changes after Irradiation

# STUDIES ON MITOCHONDRIA

## I. THE CHANGES OCCURRING DURING EXPERIMENTAL THYROID HYPERPLASIA AND ITS INVOLUTION WITH IODINE*

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According to Grynfeltt [1] the mitochondria in the thyroid were first studied specifically by O. Schultze [2] in the rat. Later they were described by Mawas [3] in the rabbit, and by Grynfeltt [1] in the horse, ox, sheep, and kitten. In all the animals studied the findings were essentially similar. In the "normal" glands they found variable numbers of granules, rods and filaments, the latter oriented parallel with the long axis of the cell. They also noted that the mitochondria were usually more numerous near the lumen surface of the cell. Attempts were made to associate the variations in the number and shape of the mitochondria with alterations in the specialized activities of the cells, as for example the production of secretion, participation in colloid formation, and other possible physiological functions.

In the light of the recent literature, it appears very unlikely that mitochondria are concerned with the specific activities of cells, but they may, perhaps, participate in the basic cellular activities such as metabolism and respiration.

It is safe to assume that "even if the mitochondria are as inert as iron filings their presence in such variable amounts must surely exert some influence upon the activity of the cells containing them, and we have good reason to think they are not chemically inert substances" (Cowdry,[4] p. 82). Moreover, there is a growing conviction that "variations in the shape and size of mitochondria constitute by far the most delicate criterion of cell injury known to us. Mitochondria react long before the nucleus, and their morphology is the first thing to change, though it is soon followed by alterations in distribution and in amount" (Cowdry, [4] p. 71).

Our findings on the variations in number, form, and distribution of mitochondria in the thyroid cell may modify some of the current

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opinions regarding the anatomical evidences of thyroid "activity" and have some bearing on the question of morphological indicators of cellular activities in general.

In 1916 Goetsch [5] reported that in the thyroid adenomata from cases diagnosed "toxic adenoma" the mitochondria were increased in number over the normal, although with ordinary methods of fixation and staining the sections failed to reveal any evidence of over-activity, i. e., hyperplasia. Upon the assumption that this increase is associated with an increased activity (secretion) of the cells he believed that there was a correlation of the clinical manifestations of thyroid hyperfunction with the anatomical changes in the gland — when the mitochondria were used as the criterion of activity. According to Cowdry [4] (p. 136), "the correlation of the clinical manifestations of hyperthyroidism and the mitochondrial picture is such that Goetsch is justified in saying that the two are definitely related. His conclusions are based upon the study of approximately 125 cases of thyroid disease in the human." Concerning the significance of Goetsch's discovery, Cowdry [4] states (p. 44), "It has come to be an embarrassing question to ask one to pick out from a number of thyroids, on the basis of the histological appearance, the one which was associated with the clinical symptoms of hyperthyroidism. The height of the epithelium, the appearance of the nuclei, and the amount of colloid are, at best, but poor aids in the dilemma. Now Goetsch has discovered that, in the cases which he has observed, the mitochondria are enormously increased in number where there are symptoms of hyperthyroidism. In other words, he has succeeded in correlating the perplexing clinical symptoms in the little-known condition of exophthalmic goiter, or Basedow's disease, with a definite anatomical change in the gland itself (i. e., in the mitochondria)."

Key [6] and Bolt [7] have reported similar increases in the number of mitochondria in pathological thyroids. The latter emphasized that this finding in the "colloid goiters" established the anatomical basis for the clinical symptoms of hyperactivity of the gland in the absence of the commonly sought for hyperplasia.

Working with routine histological methods, Marine showed that the anatomical variations of the thyroid were due to variations in the percentage-content of iodine in the gland irrespective of the clinical syndromes with which the variations were associated. All attempts to reconcile the anatomical variations with the functional

disturbances based upon clinical conceptions of its activity have failed. This failure, no doubt, is largely due to our incomplete knowledge of the physiology of the gland against which we are attempting to titrate its anatomical pictures. The definite association of clinical symptoms with specific mitochondrial changes by Goetsch appeared to confirm the clinical conceptions of the functional activity of the thyroid upon an anatomical basis — not evident in routine histological sections, but hidden in the finer cytological changes in the cells.

What would appear to be the best confirmation of Goetsch's work is found in the inability of Nicholson [8] to produce by various experimental procedures (except in compensatory hypertrophy) an increase in the mitochondria in the thyroids of animals, even though he resorted to manipulations calculated to alter the respiratory and metabolic activities of the thyroid cells. Many of his manipulations resulted in a decrease, and to quote him (p. 73), "In no one case has it yet been possible to bring about experimentally an increase in the length of the mitochondrial filaments — that is to say, to produce a change such as Goetsch [5] discovered in exophthalmic goiters." (We could find no mention of the increase in length of the mitochondrial filaments in the reference [5] cited by Nicholson or in any of the publications by Goetsch.)

The results of Nicholson's experiments implied that mitochondria were perhaps not so sensitive to variations in the metabolic activities of cells as previous work had suggested. Goetsch's findings implied that alterations in the mitochondrial constituents of the thyroid cells were indicative of variations in the functional activities which were associated with clinical syndromes.

The exact significance of mitochondrial variations in cells in general, and in the thyroid in particular is unknown. It occurred to us that we might arrive at a better understanding of these by recording the variations in the mitochondria, if any, which take place in the thyroid cell, when these changes could be titrated against a well-known and controllable activity of the gland. We had in mind the response of the thyroid cells to disturbances in the iodine content of the gland. This report deals with the alterations in the number, distribution, and morphology of the mitochondria of the thyroid cell during the passage of the gland from the involuted, or "normal" state, to the hyperplastic state, and vice versa.

## Methods and Materials

Though the underlying mechanism and significance of the hyperplasia are not yet clearly established, its experimental production in animals by partial removal was demonstrated by Halsted [10] and by high fat feeding by McCarrison.[11] Both have been confirmed by others. As in all other physiological hyperplasias of the thyroid, involution is brought about by the administration of iodine. We have studied the mitochondrial variations during the developmental stages of hyperplasia induced in the thyroids of cats, rats, rabbits, guinea pigs, pigeons and chickens by high fat diets, and during compensatory hypertrophy in the guinea pig, as well as those occurring during the involutionary response produced by iodine. In this paper we wish to report particularly our observations on the rat and guinea pig, although they were the same in all the animals studied.

Two rats were killed at the beginning of the experiment as controls and the remainder of a series of twenty were fed 3 c.c. of cottonseed oil daily in addition to their standard rations. By killing pairs at weekly intervals we obtained a series of glands showing the progressively increasing degrees of hyperplasia during nine weeks of the fat feeding. The changes occurring during the involution phase were studied in guinea pigs by the administration of iodine after the hyperplasia was well established.

The thyroid specimens were fixed in Regaud's solution and stained according to a slightly modified method as given by Cowdry [4] (p. 60) with acid fuchsin methyl green.

## Observations

In the "normal," low cuboidal cell the mitochondria are few in number, scattered diffusely, though slightly more numerous at the lumen pole of the cell, and are chiefly granular with occasional rods and filaments. Concomitant with the appearance of the histological evidences of hyperplasia, namely, an increased vascularity and diminution in the colloid content of the gland, and a hypertrophy of the epithelial cells there occur characteristic alterations in the number, distribution and morphology of the mitochondria in the thyroid cell. In the hypertrophic cell, the number is increased * so that the cyto-

* We are inclined to believe that the increase in the mitochondrial substance is absolute, and independent of the increase in the volume of the cytoplasm. It would be of interest to attempt a study of the mitochondrio-cytoplasmic relationship in the

plasm is actually filled with the red staining elements; there are huge condensations at the lumen or apical pole; and in form, they are almost purely filamentous. The shorter types are the transversely cut or fragmented forms of filaments. The filaments are oriented perpendicularly to the basement surface of the cell, fairly straight in the shorter forms, but distinctly wavy when they extend from the base to the free surface or are lost in the apical masses. The accumulations at the apical poles are often so dense that the individual forms are barely discernible.

When on the administration of iodine the gland begins to involute and the cells to flatten, the mitochondria begin to diminish in number, the apical clumps decrease, and granular and rodlike forms appear in the cells. In the flattened cells of the completely involuted gland only an occasional granule is visible.

## Discussion

In the hyperplastic thyroid we have found an *increase in the number of the filamentous type of mitochondria throughout the entire gland.* If the increase occurred only in the number of mitochondria this might be attributable to the fact that different portions of the gland were compared, or that different sections were unknowingly subjected to slightly varied conditions during fixation or staining, or to the fragmentation of the larger forms. It is well known that the mitochondrial elements are very susceptible to injury by technical manipulations. The conflicting opinions regarding their number and morphology in the same tissues, under apparently the same conditions, may in a large measure be accounted for by the differences in the technique used by the various workers. We have employed several different methods of fixation and staining but in our hands the Regaud fixation and a slightly modified Cowdry method of staining with fuchsin and methyl green yielded the most satisfactory specimens. We can confirm Cowdry's [4] statement (p. 64): "When fixatives do alter mitochondria, they alter them in a definite way. Filamentous mitochondria break up into granules and spherules. Filamentous mitochondria are never formed by the fixative from

thyroid cell similar to that made by Thurlow [9] in nerve cells. In the thyroid cell, where the variations in the cytoplasmic volume is experimentally controllable the results may be more conclusive and the significance of the disturbances, if any, may be more easily determined.

granules." The preservation of the filamentous structures intact is the best check on our technical methods.

*Mitochondria and Functional Activity of the Thyroid.* The thyroid cell is very labile. In any large series of animals kept under apparently similar conditions the thyroid mitochondria show marked individual variations. It is therefore obvious that such animals cannot be used as "normals" as many students of this subject have done. We involute the thyroid cells to uniformity by the administration of iodine before beginning our studies.

The inability of Nicholson to produce an increased number and elongation of the mitochondria may be due to the fact that he was unable to produce hypertrophic cells. In the hyperplasia which he obtained following partial removal, his failure to observe the increase in the filamentous forms may be due to some unrecognized error in technique. For, in a small series of guinea pigs in which we studied the changes during compensatory hypertrophy our specimens showed the same qualitative and quantitative variations, according to the degree of hypertrophy, as was described in the hyperplasias produced by the fat feeding. It is worthy of note, however, that Nicholson found that the administration of iodine or thyroid substance to his "normal" animals produced a "fragmentation" of the filaments, and a diminution in the number of the mitochondria.

The findings in the thyroid of humans are to be interpreted very cautiously for here we are confronted with the so-called "adenomata," the formation and significance of which do not concern us here. When once formed, however, Marine [12] has shown that their response to the administration of iodine is abnormal. While the remainder of the gland may be involuted the adenomatous tissue may persist in its hyperplastic state, or under conditions bringing about hyperplasia of the normal portions of the gland, the adenomatous structures remain unchanged. The significance of this fact is unknown, but it enables us to attempt an explanation of the findings in the human material. The presence of the large numbers of mitochondria in the non-hyperplastic adenomatous tissue observed by Goetsch and Bolt may be accounted for on the assumption that the cells had either just begun to hypertrophy or that the flattening had just occurred, and that the mitochondrial delay was an indication of the tardy response of the cell to influences which had produced the involution. If it could be demonstrated that the response of the

mitochondria becomes evident before or persists longer than the changes which occur in the remainder of the cell, the possibility that these adenomatous structures are indicative of some form of activity not shared with the remainder of the gland might be granted. It is not likely, however, that this difference would be detectable by clinical symptoms. For, with the development of the hyperplasia and its involution by iodine, during which the mitochondrial variations take place, there were no clinical evidences of disturbances in the functional activity of the thyroid that we were able to recognize. We may add also, that we have seen specimens of glands from cases of exophthalmic goiter showing the involuting effects of the iodine which had been administered therapeutically, and in spite of the marked reduction in the mitochondrial contents of the cells there was no parallel decrease in the severity of the clinical symptoms.

The final proof that an increase in the number of mitochondria in the thyroid is not associated with an increased functional activity based upon clinical symptoms will be given when it is demonstrated that in cretinism and myxedema, in man and in animals, the mitochondria in the hypertrophic thyroid cells are similar to those described in the hypertrophic thyroid cells of our experimental animals.

*Mitochondria and Cellular Activities.* The true significance of the mitochondrial variations in the thyroid cell may remain unknown until the relationship between the morphology of the thyroid and its iodine metabolism becomes established. Nevertheless, it is apparent that a single though definite "activity" of a highly specialized cell has been correlated with definite and striking alterations in its mitochondrial contents. The specific affinity of the thyroid cell for iodine which enables one to produce experimentally alterations in its mitochondrial constituents may offer a suitable means of studying certain problems.

It is beyond the scope of this communication to enter upon a discussion of the various theories regarding the relationship between the mitochondria and cellular activities in general. They have been associated with every conceivable biological activity, from forming the cytoplasmic basis of organic inheritance to participation in histogenesis and in the specialized activities of highly differentiated cells — such as contraction, secretion and conduction. Goetsch looked upon them as indicators of clinical syndromes. Others have regarded

them as bacterial symbiotes of cells. The conflicting evidence for and against these views is outlined in the extensive monograph by Cowdry.[4] For the present we may state that most of the evidence was deduced from the alterations in the number, form, and distribution of these elements under the conditions investigated.

It is quite possible that the conflicting results under the same conditions are due to unrecognized errors or variations in the technique employed by the different observers. We are now enabled to study accurately some of the factors concerned with the preservation and disintegration of the filamentous forms in fixed tissues by checking the technical variants against the mitochondrial pictures in the hypertrophic thyroid cell, in which we know that the mitochondria should appear as large numbers of the filamentous type.

Regardless of what the significance may be the fact is that in the thyroid cell the increase in the volume of the cytoplasm and nucleus is accompanied by an increase in the number and length of the mitochondria. As yet we have been unable to determine whether the changes take place first in the nuclear, cytoplasmic or mitochondrial constituents, and whether in the latter the number, morphology or distribution is first to be altered.

The ability experimentally to alter the distribution of the mitochondria may offer a suitable method for studying their relationship to cell polarity and the secretion and absorption of the colloid in the gland. It is evident that the largest amount of mitochondria is present in the thyroid cells when the colloid content of the gland is least. This fact may have some bearing on the chemical processes in the gland and on the question of the chemical constituents of mitochondria.

## Summary and Conclusions

The number, morphology and distribution of the mitochondria in the thyroid cell vary directly with the degree of active hyperplasia or involution. During the developmental stages of active hyperplasia the mitochondria increase in number, become filamentous and concentrate at the lumen pole of the cell. During involution the mitochondria decrease in number, become shorter and more scattered.

The cycle of histological changes in the thyroid has been shown by Marine to be dependent upon variations in the percentage-iodine

content. Our studies on mitochondria indicate that the variations in these elements also depend upon the same factor, and therefore cannot be correlated with existing clinical classifications of thyroid diseases.

The relationship between variations in the mitochondrial constituents and the functional activities of cells is unknown. The specific affinity of the thyroid cell for iodine may offer a suitable method for inducing controllable mitochondrial alterations by which future studies may lead to a better understanding of the significance of such variations, and possibly the relationship between mitochondria and cellular activities.

The writer wishes to acknowledge his indebtedness to Dr. David Marine for his valuable assistance throughout the progress of the work.

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# THE ORIGIN OF THE MONONUCLEAR PHAGOCYTES OF PERITONEAL EXUDATES *

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That the chief source of the granular leucocytes is the bone marrow has been definitely established. Also lymphocytes of the usual type without doubt are derived from lymph glands and elsewhere from lymphoid tissue. Much investigation of the group of mononuclear phagocytes found in the blood and extravascularly has not so far resulted in a satisfactory explanation of the origin and relationship of cells of this type. Two prominent views of the origin of such phagocytes are those of Maximow [1] who holds that they are derived from a mesenchymal cell, the "hemocytoblast," that has the ability to produce all blood and wandering cells, and of Mallory [2] who traces their origin to vascular endothelium. These two views in regard to the origin of the wandering phagocytes are referred to especially because they are conclusions arrived at in two essentially different ways: Maximow's observations were made on embryonic tissue and the tissue of adult lower animals in cultures and in fixed and stained specimens; Mallory's, on the behavior of human tissue subjected to various pathological influences.

Sabin [3] has supported the endothelial origin of the phagocytes but more recently Cunningham, Sabin, and Doan [4] conclude that these cells are derived in part from the reticulo-endothelium, in part from a specific mesenchymal cell which they found to be present in the splenic pulp. The author [5] investigating the mononuclear phagocytes of the blood devised methods for using carbon in suspension to mark the vascular endothelium and found that carbon-laden endothelial cells may undergo mitosis to become free in the blood stream. Foot [6] in a series of seven articles has described the behavior of cells of endothelial origin to carbon, colloidal dyes, and tubercle bacilli and has placed emphasis on the importance of ingested carbon in the identification of the phagocytes.

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Second only to the cells of the blood the free cells of the serous cavities, especially of the peritoneal cavity, have received the attention of experimental investigators. The author first became interested in the determination of the reaction to benzidin of the peritoneal cavity cells and later undertook an examination of them by the reaction to carbon in suspension, and by the methods of vital and supravital staining. Cunningham [7,8] in two papers has defined the characteristic features of the mesothelial serosal cells of the peritoneum. Sabin, Doan, and Cunningham,[9] examining peritoneal exudate cells by the method of supravital staining, found the phagocytic ones to consist of two distinct types. In their "macrophage" type the neutral red of the stain was taken up by the cells as large irregular granules scattered about in the cytoplasm, while in the second or "monocyte" type, which in the peripheral blood was identified as the "transitional" leucocyte, the neutral red was seen as granules arranged in the form of rosettes. The "rosette" type of cell was found in the spleen in large numbers and on this basis it was regarded by Cunningham, Sabin, and Doan [4] as arising from a "stem-cell of mesenchymal origin which is resident in the pulp." This view of the duality of the mononuclear phagocytes is at variance with the one previously expressed by Sabin [3] in which all of these cells were traced to the reticulo-endothelium.

The peritoneal exudates investigated by the author were produced by injections of India ink, trypan blue solutions, and whole rabbit's blood into the peritoneal cavities of normal rabbits, splenectomized rabbits, and rabbits receiving intravenous injections of India ink. In a routine examination of these exudates by the benzidin peroxydase method of the author [10] it was found that none of the mononuclear phagocytes reacted, the reaction being given only by the leucocytes with neutrophilic and eosinophilic granules. It may be remarked that benzidin-reacting mononuclear phagocytes are not found in the peripheral blood of the normal rabbit although they are quite abundant in peripheral human blood.[11]

*Exudate by injection of trypan blue.* This dye in amounts of 20 c.c. of a 2 per cent solution was injected into the peritoneal cavity of rabbits first for the purpose of producing an exudation of cells, and second for the vital staining of the tissues of the animals. After

intervals of about ten days, second and third injections were given. Exudate for examination was obtained by means of a syringe and fine needle, and examined at once with oil immersion lens. On the first day after first injections, polymorphonuclear leucocytes were quite numerous, but at the end of forty-eight hours the mononucleated cells predominated and of these an occasional one contained coarse blue granules. After second injections, the number of cells with coarse dye granules became greater. Preparations of exudate were examined on unfilmed and filmed slides. The dye films were made on slides by the method for supravital staining of Pappenheim [12] and Simpson.[13] This method was later made use of by Sabin, Doan, and Cunningham.[9]

A very small droplet of the exudate is pressed out from the syringe needle upon a cover-glass rimmed with vaseline and at once inverted and pressed out upon a slide covered with the dye film. The film of dye on the slide is made from a fresh mixture of equal parts of 95 per cent alcohol one-half saturated with neutral red (Gruebler) and of 95 per cent alcohol one-sixth saturated with Janus green (N. A. C. Co.). Several preparations should be made on slides with films of varying thickness for the reason that exudates with many blood corpuscles absorb more of the dyes. The lymphocytes and polymorphonuclear leucocytes are readily distinguished in the supravitally stained preparations. Both are small and although the latter have neutral red granules, the nuclear masses with granulation between them can be seen. The large trypan blue granules of the vitally stained mononuclear cells are soon stained with the red and assume a deep purplish tinge. In the vitally stained rabbits, omission of the Janus green gives better preparations. A majority of the mononuclear cells present contain numerous neutral red granules in the cytoplasm without any definite arrangement. There are occasional cells with the neutral red granules massed in a focal area of cytoplasm in the form of a rosette. The last two types were first described by Sabin, Doan, and Cunningham [4, 9] and are discussed at length in connection with the exudates obtained by the introduction of whole blood. For convenience of description the trypan blue cell may be spoken of as the "hyaline" type, the one with diffuse neutral red granules as the "non-rosette" type, while the third variety has been called the "rosette" type.

*Exudate by injection of India ink.* The India ink (Higgins') is injected intraperitoneally in amounts of 5 c.c. On the first day polymorphonuclear cells are numerous but after forty-eight hours mononucleated cells predominate. Examination by supravital staining shows a few "rosette" cells which usually contain one or more carbon particles. The "non-rosette" type well-filled with scattered neutral red granules predominates. The cells of this type have a few carbon granules. The "hyaline" type cells are also quite numerous. These cells have much the greatest capacity for phagocytosis of carbon with some cells containing a half-dozen granules and others fully packed with the carbon. In general this type heavily loaded with carbon tends to stain relatively little with the neutral red but after contact with the dye for a half hour a considerable number of irregular neutral red granules appear in the cytoplasm. Mitoses in both the "hyaline" and the "rosette" types have been seen in preparations made at the end of forty-eight hours.

*Exudate by injection of whole rabbit's blood.* This is the method of producing peritoneal exudate employed by Sabin, Doan, and Cunningham,[9] and has the advantage that it causes a marked exudation of the "rosette" type of cell. Ten to 20 c.c. of whole rabbit's blood are injected daily into the peritoneal cavity of a rabbit. By drawing 2 per cent citrate into a sterile syringe and forcing it out preliminary to the heart puncture a somewhat longer interval for the puncture and injection may be obtained. Many observations have been made on eight normal rabbits and two splenectomized ones. So far as concerns the mononucleated cells reacting strongly to the neutral red, namely the "rosette" and the "non-rosette" types, they were found to correspond in most particulars to the description of them originally given by Sabin, Doan, and Cunningham.[9]

In exudates containing large numbers of the "rosette" type of cells it has been constantly observed that there are many with neutral red granules massed in some portion of the cell but lacking the typical rosette arrangement. These forms, which morphologically appear to be transitions between the two types, lead one to think that the "non-rosette" may be an older, more differentiated form of the "rosette" cell. There is also a correspondence of other characters discussed in connection with the intravenous injection of carbon and the incubation in vitro with carbon suspensions. In the

lymph node where the "rosette" cell develops from a cell with only a minute granule of the neutral red there is evidence of an increased ability of the older cells to take up the dye. Sabin, Doan, and Cunningham, as previously stated, considered the "rosette" and the "non-rosette" type to be transitional blood leucocyte and macrophage respectively. The third or "hyaline" type of cell was not described by them.

Since the three types of phagocytes are constantly present in the exudate after a few intraperitoneal injections of whole blood, a single description will serve to illustrate their character. Many observations of peritoneal exudates by supravital staining were made on two splenectomized rabbits. On September 25, October 2, and October 6, 1924, one of these animals received 3 c.c. of India ink (Higgins' intravenously in order to mark the phagocytic endothelium. On October 7, it was injected with 15 c.c. of whole rabbit's blood into the peritoneal cavity and 3 c.c. of India ink into the ear vein. On October 8, the cells of the peritoneal exudate were chiefly polynuclears (10 per cent containing carbon) but there were a few "non-rosette" mononuclears (20 per cent with carbon). No "rosette" and no "hyaline" cells were identified; injected with 20 c.c. of whole rabbit's blood. On October 9, less than one-half were polynuclear leucocytes (carbon in 5 per cent). Of the large mononuclear cells the majority were of the "rosette" type described by Sabin, Doan, and Cunningham.[9] Next in number were the "non-rosette" cells with numerous scattered neutral red granules. The latter were much more phagocytic for red corpuscles and polynuclear leucocytes. Less than one in ten of the two neutral red types contained carbon. Carbon particles were somewhat less readily found in the "rosette" cells. As is usually the case when these two types are abundant there are transition forms with the neutral red granules massed at one point or another in the cytoplasm but not in the form of a typical "rosette." There is an occasional "hyaline" type of cell and almost all of these had phagocytized a lymphocyte or carbon granules or both. Injected with 10 c.c. of whole rabbit's blood. On October 10, the number of polynuclears had diminished and the "hyaline" cells increased in number. Almost all of the "hyaline" cells contained carbon and one with six large granules was seen. Splenectomy under ether anesthesia. On October 13, injected with 15 c.c. of whole blood

into the peritoneal cavity and 3 c.c. of ink intravenously. On October 14, few polynuclears. Of the mononuclears, three-fourths were of the "rosette" type and about one in twenty contained carbon. The "hyaline" cells were as numerous as the "non-rosette" type. On October 20, intraperitoneally 15 c.c. of whole blood and intravenously 3 c.c. of ink. On October 21, exudate unchanged. On October 30, intraperitoneally, 15 c.c. of whole blood and intravenously 3 c.c. of ink. On October 31, little change in exudate. On November 3, intraperitoneally 10 c.c. of whole rabbit's blood and no injection of ink. On November 4, at least 3 "rosettes" to 1 "non-rosette." There was less than 1 per cent of these two types with carbon. As usual there was less phagocytosis by the "rosette" type.

The "hyaline" type of cell was conspicuous on November 4 and at least one-fifth of all the mononuclear cells were of this type. Although the size of these cells was variable the largest ones present in the exudate were of this type. The round or oval nucleus was relatively small and distinct in a cytoplasm that tended to be clear. In fresh films there was very little neutral red or none whatever in these cells which were apt to contain a lymphocyte or carbon particles, but after about thirty minutes irregular red granules sometimes appeared and in occasional cells red staining became quite distinct. Often the neutral red was so irregular and granular that it suggested the staining of ingested granular débris.

About one-half of these "hyaline" cells contained carbon but usually only one or a few granules. To test in vitro their phagocytic property there is placed from a syringe at the center of a cover-glass within a 5 mm. wax-pencil circle a minute drop of 1 per cent India ink in saline and to this at once is added an equal amount of exudate from a second syringe. The cover-glass is rimmed with vaseline and at once inverted over a deep well on a concave slide. After thirty minutes at 37° C. the cover-glass is lifted by its corners from the concave slide and placed in the usual way on the dye film. Abundant fibrin threads form but these do not interfere with the phagocytosis or the subsequent examination of the preparation. The picture is remarkable. Both neutral red types take up the carbon but it is the "hyaline" ones that become enormously loaded with the ink particles. These cells are extremely ameboid and tend to agglutinate so that they are found more abundantly entangled in the fibrin toward

the center of the preparation. On this date 10 c.c. of whole blood was injected intraperitoneally and the phagocytic experiment repeated on November 5 with the same results. This exaggerated property of carbon phagocytosis has been demonstrated in several dozen exudates obtained at different times and from different animals. On November 10, 15 c.c. of whole blood was injected intraperitoneally. On November 12, there were about 3 "rosettes" to 1 "non-rosette" to 1 "hyaline." Although the cells were very numerous, only 4 "rosettes" and 5 "non-rosettes" containing carbon were found during one hour. The percentage of the "hyaline" type with carbon has diminished since the last intravenous carbon injection but carbon-containing cells of this type are much more numerous than of either of the other varieties.

### Origin of the Phagocytes in Exudates Produced by Injections of Trypan Blue, India Ink and Whole Rabbit's Blood

The origin of the three types of peritoneal exudate cells has been investigated by an examination of tissues and organs from normal, trypan blue, and India ink rabbits, first, in fresh preparations supravitally stained, and second, in paraffin sections of supravitally stained tissues.

#### *Examination in Fresh Preparations of Tissues Supravitally Stained.*

*Peritoneal Serosa.* Peritoneal scrapings made with a knife are placed on a cover-glass ringed with vaseline which is at once inverted on the dye film. Seventy-two hours after the injection of 5 c.c. of India ink there are in the scrapings abundant placques which consist of many or a few cells and in these are seen all cell shapes varying from the flattened non-irritated cell to cells that are almost perfectly spherical, but still attached to other cells. The cytoplasm is finely granular with indistinct cell outline while the nuclear membrane of the nucleus which tends to be relatively large is very distinct. By far the most conspicuous feature is the large and uniform content of very minute carbon particles. In some cells the carbon dust is massed somewhat at one side of the nucleus where the cytoplasm is denser, while in others it is scattered diffusely throughout the cytoplasm. The serosal cells in fresh preparations contain no neutral red but after a half-hour the denser portions of the cytoplasm may become slightly tinged.

The peritoneal surfaces are scraped after second and third injections of 20 c.c. of 2 per cent trypan blue. The serosal cells contain only small amounts of the dye or none at all.

On November 18, 1924, the splenectomized animal described above was chloroformed and peritoneal scrapings were examined in supravital films both before and after incubation with carbon. The phagocytic experiment with carbon was made in the same way as in the case of the exudate. The serosal cells contained no carbon and did not phagocytize carbon in vitro. The other characters of these cells have been described by Cunningham.[7, 8]

*Lymph Nodes.* Bits of organs and whole lymph nodes are kept in Locke's solution until minute snippings can be teased to dust-like particles in a solution made by adding 1 to 4 drops of 0.5 per cent neutral red to 10 c.c. of saline. A cover-glass is applied to the preparation in the neutral red solution. The second method of examination consists of expressing the "juice" from the lymph node on a cover-glass and at once inverting it on a dye film. The expressed "juice" of glands from normal animals shows such enormous numbers of the "rosette" type that entire fields consist almost entirely of this type. In the teased preparations the "rosette" cells in places are attached and have an average size larger than the lymphocytes which do not react to the dye. The neutral red spherule is in almost all of the cells smaller than in the peritoneal exudate "rosettes." In some oblong cells the neutral red focus may be seen to lie at the side of the nucleus, midway in the cell. Phagocytic ones are very rarely seen. Unlike the lymphocytes the nuclei of these cells present an irregularity of outline. Besides the lymphocytes and "rosette" cells there are occasional larger ones with abundant heavy neutral red granules. In the lymph nodes of ink-injected rabbits there are only occasional carbon-containing phagocytes. Most of these are found toward the capsule of the gland, are large and may or may not contain irregular neutral red particles. In rabbits stained with trypan blue the lymph node preparations are the same as the normal except that there are occasional large phagocytes filled with coarse dye granules which tend to take up the neutral red to assume a purplish color. The carbon-marked and trypan-blue containing cells appear to correspond to the "hyaline" type of phagocyte seen in the peritoneal exudates. The "non-rosette" type is present in scant numbers.

*Spleen.* In normal uninjected rabbits the "rosette" cells have never been observed in large numbers in material obtained by splenic puncture under ether, by puncture after removal of the spleen from the body, or by teasing snippings of pulp or of the splenic lymph nodules. The "non-rosettte" cells are fairly abundant in normal rabbits. In rabbits being injected intraperitoneally with whole rabbit's blood the "rosette" cells are much more numerous. In the India ink rabbits the numerous carbon-marked phagocytes may show irregular neutral red staining or none at all. In the trypan blue rabbits there are numerous large phagocytes containing irregular granules purple in color from imbibition of the red dye by the trypan blue granules.

*Liver.* Teased preparations of the ink-injected rabbits in the neutral red-saline solution show heavy masses in the sinusoids consisting of carbon-laden cells. These cells take up little of the dye but at the periphery of these masses mononuclear cells with scattered red granules are seen in small numbers. The sinusoidal endothelial cells show neutral red granules beyond the ends of the nuclei. In rabbits stained with trypan blue there is a much more distinct granulation in endothelial cells which takes on a purple color in the dye films. In normal rabbits irregular neutral red particles may be demonstrated in an occasional endothelial cell.

*Bone-Marrow.* The less mature myelocytes may have the neutral red granules grouped in a way that suggests the "rosette" cells, but these masses are usually not spherical. In the normal rabbit positive identification of "rosette" cells has not been possible. In the India-ink rabbits the bone-marrow becomes quite dark and large ink-containing phagocytes as well as polynuclear leucocytes with carbon are present. The former show a few coarse particles of neutral red.

*Examination in Paraffin Sections of Tissue Supravitally Stained.*

It soon became apparent in examining teased bits of tissue that, in order to obtain contact with the dye, it was necessary to destroy to a very large extent cell relations. In fact observation of the fresh tissue proved entirely inadequate for an exact determination of the origin of the cells reacting to neutral red. The method for imbedding tissue supravitally stained is one previously developed by the author [14] for the preservation in paraffin sections of the cytoplasmic granules giving the peroxydase reaction to benzidin. The tissue

stained supravitally in toto is fixed for 12 hours in a Zenker-formol solution consisting of 15 c.c. of 40 per cent formaldehyde and 85 c.c. of Zenker's fluid without acetic acid. It is then cut into pieces not to exceed 3 mm. in thickness and transferred to Zenker's fluid without acetic acid for 12 to 24 hours. Bits of the tissue are then placed in pure absolute acetone for 1 hour (2 changes), in benzol for 20 minutes and in 52°C. paraffin for 20 minutes. Sections of the desired thickness are cut and attached to the albumin-coated slides by allowing them to dry overnight at room temperature. To stain, the paraffin is removed with xylol (10 seconds) and pure acetone (10 seconds). After immersion in water (5 seconds) the slide is stained very lightly with hematoxylin (Harris without acetic acid) for about 5 seconds and immersed in tap water (5 seconds). The section is then dehydrated with pure absolute acetone for 10 seconds, at once immersed in xylol for 10 seconds and mounted in balsam. To limit the action of the acetone and xylol to these times the slides are stained singly and the solutions run over them from a dropping bottle. A 0.05 per cent aqueous solution of methylene blue may be substituted for the hematoxylin.

To stain the tissue supravitally in toto the animal is bled completely from the carotids under light ether anesthesia and saline solution saturated with neutral red (Gruebler) is at once injected directly into the tissues. About 0.5 c.c. is injected into the liver or spleen by inserting a fine needle 2 cm. from the edge of the organ and slowly forcing out the dye after carrying the needle to within a few millimeters of the margin of the organ. The injection is repeated in 15 minutes. The inguinal, iliac, substernal and axillary lymph glands are injected by inserting the needle into the substance of the gland and forcing out the dye until the gland is red and distended. After about 30 minutes the injected tissues are removed, covered with cotton moistened with saline and after another 30 minutes at room temperature placed in the Zenker-formol. It is always advisable to imbed all the available tissue, for the contact with the dye is irregular. Similar results have been obtained in rabbits and guinea pigs. A larger number of guinea pigs have been examined and for this reason some of the best reactions have been obtained in them. The nuclei of the cells that are killed by the injection stain red, and no observations are made within the areas in which the cells are injured.

*Lymph Node.* In the normal lymph gland both "rosette" and "non-rosette" cells are abundant and both are found especially in the sinus ramifications toward the hilum. Few neutral red cells occur within the larger masses of lymphoid tissue such as the secondary nodules although they are present at the periphery of these structures. That the dye has penetrated this dense lymphoid tissue is shown by the presence there of occasional neutrophiles or blood vessel endothelium with dye granules. The anastomosing lymph sinuses when collapsed consist of connecting cells which as usual are not to be differentiated from reticular cells. They are larger than lymphocytes and tend to be elongated or oval. The nuclei are not spherical and frequently an actual hof is present. These are the cells that react to the dye. In areas in which the dye penetration is good with several dozen "rosette" cells present in one microscopic field the idea of the very great number of these cells is obtained. The flat cells with scant cytoplasm that line the peripheral and larger sinuses are usually devoid of dye but the reaction may be identified in definite endothelium lining medullary sinuses. These channels are apt to be broadened by the injection and may be followed for considerable distance in longitudinal section. Where they are surrounded by more compact masses of lymphoid tissue no lumen can be seen. With this arrangement rows of reticular cells are seen, each with its spherule of neutral red. Here and there the larger of the reticular cells present a perfect rosette appearance. In these larger cells one may occasionally observe two or more foci of fine granules but it is usually within the lumina of the larger sinuses that cells with several foci of minute granules occur where there are also cells with the fine granules distributed throughout the cytoplasm. All transitions are present from the reticular cells with a single small collection of fine granules to those with rosettes and with numerous scattered red granules. There is, however, unquestionably a tendency for the neutral red granules to remain in foci. In rabbits given intravenous injections of ink these cells are devoid of carbon but large carbon-containing phagocytes are present in small numbers in the sinuses and especially in the fibrous capsule of the gland. These carbon-phagocytes show neutral red granules irregularly distributed when they are brought into contact with the dye. In rabbits injected with carbon suspension subcutaneously the reticulo-endothelium of the regional lymph nodes contains carbon particles but

these cells do not usually become engorged to form great ink masses like those seen in the liver or spleen of rabbits intravenously injected. The small foci of fine neutral red granules are present in young reticular carbon-containing cells of the subcutaneously injected rabbits. The ink granules are not grouped in rosette forms by these cells. It is advisable to stain six or more lymph nodes for both neutral red and carbon reactions vary. The lymph nodes draining such ink foci offer perfect evidence that the reticular cells are reached by the lymph although the channel that they line cannot be seen in the usual section.

The view that any distinction between the lymphocytic endothelium and the reticular cells is arbitrary was first advanced by Aschoff and Kiyono.[15] They hold that the two are indistinguishable and function in the same way. They make no distinction as regards function or morphological features between the reticulo-endothelial apparatus of the spleen and that of the lymph nodes although one carries blood and the other lymph. Their conclusions are based largely on results obtained with vital dyes. Maximow,[1] on the other hand, considers the reticular cells to be a type entirely distinct from the vascular endothelium.

*Spleen.* An intravenous injection of 1 c.c. of India ink several days before the test is helpful in defining the structures of the spleen where even under the best conditions of fixation and staining, or even in specimens with injection mass in the blood vessels, the vasuclar network in its fine ramifications in the pulp and lymphoid tissue is not easy to distinguish. In places the endothelium of the vessels contains fine red granules, but mononuculear cells which lie within and between the well-defined sinuses and which have a conspicuous fine red granulation scattered diffusely in the cytoplasm are especially abundant. These numerous phagocytes with a very fine diffuse granulation appear to be free or semi-detached. Some of them contain carbon, red corpuscles or leucocytes. In the guinea pig Kurloff bodies in the lymphocytes are likely to stain supravitally and to be conspicuous. In these preparations the neutral red granulation does not aid greatly in distinguishing these cells from myelocytes, but in control oxydase stains myelocytes are not abundant. The granulation of these mononuclear phagocytes appears to be brought out only by a relatively high concentration of dye, but as in the case of other tissues cell injury is shown by nuclear reaction to

the neutral red, and the cytoplasm of such injured cells does not show the granulation. These numerous free cells have much the same appearance as the larger attached endothelial cells, but it is highly important to recognize the ease with which mononuclear cells circulating in the blood are arrested in the spleen. The "rosette" cells are relatively infrequent and the young forms of "rosette" cells with single foci of neutral red granules have not been seen in the pulp or at the periphery of lymphoid nodules where dozens are present in single oil-immersion fields in the lymph nodes.

*Liver.* It may be necessary to examine several blocks from different parts of the injected bit of tissue in order to find an area that has reacted well. The Kupffer cells and other endothelial cells lining the sinusoids which have a notable amount of cytoplasm may contain neutral red granules irregular in shape and size, but in well-stained foci others of these cells contain no dye. Some of the granules have the irregular appearance of ingested material that has taken up the stain but others are fine and regular. The mild irritation of a preliminary intravenous injection of 1 c.c. of India ink causes some increase in the size of the endothelium without obscuring it by overloading with carbon. In the elongated cells the dye granules may be present only at one end of the nucleus but more often are situated at both ends. In the larger endothelial cells and the Kupffer cells the dye granules are almost always scattered about in the cytoplasm much like the ink particles. Free within the sinusoids large phagocytes with carbon and dye in abundance are found.

## Discussion

The polymorphonuclear leucocytes, lymphocytes, and the desquamated serosal cells which appear in peritoneal exudates are quite readily identified in fresh preparations supravitally stained with neutral red and Janus green. Following injections of whole rabbit's blood into the peritoneum there appear in the peritoneal cavity three types of mononuclear phagocytes which have for convenience of description been designated the "hyaline," the "rosette," and the "non-rosette" types according to the amount and arrangement of neutral red in them.

The "hyaline" cells vary in size from about 20 microns to very large cells. The nuclei tend in the larger ones to be relatively small

and to lie excentrically in the glassy cytoplasm. These cells are actively ameboid and frequently contain ingested lymphocytes and especially amorphous material that takes up more or less of the neutral red. Some cells have irregular dye granules while others have none. They become more abundant after many injections of whole blood. They are clearly defined by incubation with carbon suspension since they adhere to the fibrin threads where they form agglutinated masses of phagocytes containing enormous quantities of ingested ink particles. In rabbits that have the vasuclar endothelium marked by the intravenous injection of India ink more than one-half of this type of cell appearing in the peritoneal cavity after the introduction of whole rabbit's blood may contain one or two carbon particles, but cells with great masses of carbon such as those present in the capillaries of the spleen and liver rarely appear in the exudate. When whole rabbit's blood is injected into rabbits stained vitally, a considerable number of the cells of this type contain the coarse trypan blue granules. The reactions of this type of peritoneal phagocyte to carbon and to some extent to neutral red are essentially the same as those of the blood vascular endothelium seen in fresh preparations and in paraffin sections of tissue supravitally stained. The presence in these cells of carbon and trypan blue granules is evidence favoring their vascular origin.

The "rosette" and the "non-rosette" types were originally described by Sabin and co-workers.[4, 9] By them the "rosette" cell was considered to be the monocyte or transitional cell of the peripheral blood and to be derived from a mesenchymal cell in the splenic pulp while the "non-rosette" cell was regarded as the "macrophage" of reticulo-endothelial origin. The observations of the author in fresh tissue and in paraffin sections of supravitally stained tissue of normal and splenectomized rabbits indicate the lymph node origin of the "rosette" cell. In paraffin sections of supravitally stained lymph nodes the reticular lymph vascular endothelial cells lining the medullary lymph sinuses and forming the complex anastomosing channels about the hilum of the gland and in the periphery of the follicles of lymphoid tissue contain cytoplasmic foci of dye granules or dye granules in actual wreath form. In some of the larger cells there is more than one focus of dye granules. Within the larger lymph sinuses there are free cells with dye granules in the form of rosettes and with dye granules scattered about in the cytoplasm. There is,

however, a tendency for the granules to remain more or less circumscribed in certain portions of the cytoplasm. That is, in the attached reticular cells the dye-reacting cytoplasm may progress to the "rosette" form but the rosette formation and any further dissemination of the supravitally staining granules usually takes place after the cells separate or at least after they assume a semi-detached position in the sinuses. It appears therefore that phagocytes originating in the lymph nodes as well as those arising from the blood vascular endothelium may present neutral red granules scattered diffusely in the cytoplasm, but that the granules are always scattered in the blood vascular ones while they tend to remain in foci in those of lymph node origin. There is therefore satisfactory evidence that the neutral red granules may become dispersed in the cytoplasm with a change from rosette to non-rosette form.

In rabbits injected simultaneously with ink into the ear vein and with whole rabbit's blood into the peritoneal cavity the relatively large percentage (10 to 20 per cent) of "rosette" and "non-rosette" cells with phagocytized carbon is regarded as evidence that these cells are transported in part at least through the blood stream. The percentage of carbon-containing polymorphonuclear leucocytes is about the same. At the end of 4 days less than 1 in 100 of the "rosette" cells contains carbon, although ink granules may be present in one-half of the "hyaline" cells at this time. Since the blood vascular endothelium is carbon marked, the tendency for carbon-containing "hyaline" cells to persist and be present at a time when few granular leucocytes and few mononuclear neutral-red types contain carbon is significant. In rabbits moderately stained with trypan blue a small number of cells with coarse dye granules appear in the exudates produced with whole blood and when supravitally stained such cells take up neutral red so that the dye granules assume a purple color. In the lymph nodes of the animals vitally stained to a moderate degree with two or three injections of trypan blue there are a few large phagocytes in the capsule and in the peripheral sinus of the gland which contain coarse dye granules but the abundant "rosette" cells of the lymph node at this time contain none. In the adult rabbits no evidence was obtained indicative of a form of the "rosette" cell younger than the reticular endothelium which is present in the lymph node and which freely undergoes mitotic division to form its own kind.

After completion of this article but before its submission for publication, Sabin and co-workers [16, 17] have given a much more detailed presentation of their work. The illustrations are especially helpful. In Figs. 5, 6, and 13 of Plate I, portraying clasmatocytes, of the article by Sabin, Doan, and Cunningham [16] there appear to be none or very few neutral-red granules in the cell cytoplasm. In their earlier communication the clasmatocytes or macrophages were described as follows: "They were usually large cells, 15 to 30 micra in diameter. These cells always reacted intensely to neutral red; the aggregations of the stain varied greatly in size and color (slight red to deep maroon) and were scattered irregularly, without any pattern, throughout the cell." From an analysis of the text and illustrations the inference is made that their clasmatocytes correspond to the author's "hyaline" type. They apply the term "wandering endothelial phagocyte" to the clasmatocyte and regard it as the derivative of the vascular endothelium. It is therefore chiefly in regard to the monocyte or "rosette" cell that their view differs from that of the author, since they state (p. 158): "Thus we consider that the weight of evidence points to two separate strains of phagocytic cells of connective tissue: clasmatocytes, which are of endothelial origin and come into the blood stream only occasionally and abnormally, and monocytes, which are a constant type of blood cell, arising, largely in the spleen, but also a specific cell of the diffuse connective tissues, where they both arise and function." The examination of lymph glands made by Cunningham, Sabin, and Doan [17] gives results emphatically different from those of the author. Examination of the fluid obtained by puncture of mesenteric lymph glands gave them results as follows (p. 259): "Over 90 percent of the cells obtained from such a puncture, especially if the pipette does not rupture any blood vessels, are unquestionably members of the lymphocytic series. The other 10 percent are made up of a few clasmatocytes, a few polymorphonuclear leukocytes, and an occasional myelocyte. . . . In no experiment where blood vessels were not ruptured by the puncture did we find any typical young monocytes." Lymph nodes have not been punctured with the fine capillary pipettes used by these observers but it is scarcely conceivable that cells free from the "rosette" type could be obtained unless a very fine capillary entered a lymph follicle. In juice expressed from inguinal, iliac, and axillary lymph nodes, cells with single focal areas

of neutral red granules including rosettes at times number one-third of all cells in the preparation. In paraffin sections, as described, these cells are very numerous and correspond to the reticular endothelium.

Cunningham, Sabin, and Doan reach the final conclusion: "that there are two fixed-tissue stem-cells from which all the blood cells arise. From the endothelium the red blood-cells and the clasmatocytes are formed, and from the reticular cell there is formed a primitive stem-cell which in turn gives rise to the polymorphonuclear leucocytes, the monocytes, and the lymphocytes." The impression has been gained by the author from observations on adult tissues that the phagocyte of the "hyaline" type which appears usually to have been designated as macrophage, histiocyte or clasmatocyte, arises from blood vascular endothelium, while the phagocyte presenting the fine nuetral red granules, often in the form of a rosette, takes its origin from the reticular lymph vessel endothelium which is in the adult tissues a cell distinct from the lymphocytes and younger lymphoblastic cells at the germinal centers. The evidence supporting the lymph-node origin of the "rosette" type is considered to be conclusive. In the preparations from which these conclusions are drawn the lymphocytes show not the slightest trace of neutral red. With a dissemination of the granules and loss of the characteristic wreath appearance the older cells of this type are not so readily identified in supravital stains, and they assume the form designated by the author in this paper as the "non-rosette" form and by Sabin and co-authors as the "macrophage" cell. That all of the cells of the "non-rosette" group of the author are of this type however seems doubtful since the "hyaline" form of phagocyte may show scattered neutral red granules.

Observations in animals with their vascular endothelium carbon-marked and in animals vitally stained to a moderate degree with trypan blue indicate that the "hyaline" form of the author arises from the blood vascular endothelium, but the proof is less direct than that by which the "rosette" cells are traced to the lymph nodes. In the spleen the predominant phagocytic cell is one with scattered fine neutral-red granules, and, since an investigation of the cells of this organ is essentially a study of the mononuclear phagocytes of the blood, a detailed discussion of it here will not be undertaken. It may be stated merely that it corresponds to the "non-rosette" type of the author which has a double origin. So far as

can be determined removal of the organ does not influence the character of the phagocytes appearing in peritoneal exudates.

## Conclusions

1. By the method of peroxydase staining with benzidin, reacting mononuclear phagocytes are not demonstrable in the peritoneal exudates of rabbits.

2. By the method of supravital staining the mononuclear phagocytes of peritoneal exudates may for the purpose of description be divided into three groups: "hyaline" cells with a variable affinity for neutral red; "rosette" cells with neutral red in wreath form; and "non-rosette" cells with dye granules scattered diffusely in the cytoplasm.

3. By in-vitro incubation with carbon, by the intravenous injection of carbon suspension and by vital staining with trypan blue, the "hyaline" type may be differentiated from the other cells of the exudate. In paraffin sections of supravitally stained tissue in normal and carbon-injected animals, this type of cell reacts much like the reticular blood-vessel endothelium. This evidence suggests the vascular origin of the "hyaline" type.

4. By the examination of fresh tissue and paraffin sections of fixed tissue supravitally stained, satisfactory evidence of the origin of the "rosette" type from the reticular lymph vessel endothelium is obtained.

5. The "non-rosette" type with its diffuse neutral-red granulation is a mixed group of double origin. By the methods employed all mononuclear phagocytes of this type in the exudates appear to be either "hyaline" cells of probable blood-vascular origin, which may have diffuse neutral red granules, or "rosette" cells in which the granules have become disseminated.

6. The lesser phagocytic activity for coarse carbon particles of the lymph-node phagocytes and the tendency for neutral-red granules to remain localized in foci in them offer a basis for the differentiation of a lymphatic and of a blood-vascular or hemal type of mononuclear phagocyte. Although the free phagocytes may undergo mitosis, the fixed-tissue source is the blood- and lymph-vessel endothelium. The endothelial origin of the peritoneal phagocytes

agrees with the conclusion previously arrived at by the author [5] in regard to the mononuclear phagocytes of the peripheral blood.

7. As a means for the identification of cells, further evidence of the value of their vital reaction to chemical or physical agents is seen in the application of neutral red solution to the living mononuclear phagocytes of peritoneal exudates. To obtain exact data in regard to the origin of the cells it is essential to fix the neutral red in the tissues for examination in paraffin sections.

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# STUDIES ON FILTERABLE VIRUSES *

## I. CULTIVATION OF VACCINE VIRUS

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One of us [1] in a preliminary note has reported the successful cultivation of vaccine virus in vitro. The medium employed was a tissue culture of rabbit testis. The virus was successfully carried through a maximum of nine transfers, representing a total time of fifty-four days in the incubator. The virus showed a definite increase on comparing the first and third generations of two of the cultures. We wish to report below further work along the same general lines.

### *Growth of Virus in Normal Tissue*

In the work referred to above each culture was started with pieces of fresh, vaccine-infected testis. In view of Wolbach's [2] experience with the cultivation of Rocky Mountain spotted-fever virus, in which he gained the impression that only cells originally infected in the animal, or descendants of these cells, contained the virus in cultures, we thought it of interest to attempt to discover if the same held true of vaccine virus.

For this purpose pieces of normal testis were soaked for five minutes in a suspension of glycerinated virus-infected testis, that had been kept in the ice box for seventy-one days, after which time of course no cells could be expected to survive. These pieces of testis were then covered with drops of normal rabbit plasma, diluted with two parts of Ringer's solution, incubated for five days, transferred, reincubated, etc., as described in the preliminary note. The fourth generation of this culture was tested on a rabbit's cornea and found markedly positive macro- and microscopically. This virus, VC 6 A, was carried through 36 transfers (Table I). The experiment would appear to show that the growth of this virus, unlike that of spotted fever, does not take place only in the originally infected cells, but

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occurs either in or near normal tissue cells. Other experiments carried out in a somewhat different way also confirm this conclusion. In these experiments pieces of normal testis were covered with drops of normal plasma to which had been added small amounts of a heavy undiluted suspension of glycerinated, vaccine-infected testis. Here again, after several transfers, virus could be demonstrated and shown to be growing in all of the cultures (VC 10 — Table II — represents one of these cultures).

Numerous investigators in studying vaccinia in animals and man have attempted to demonstrate the virus in the blood of the infected animal, but always without success until quite recently. Ohtawara,[3] by using intratesticular inoculations with comparatively large amounts of blood, has shown conclusively that the virus does occur in the blood stream and remains there several days following inoculation on the skin with vaccine virus. This was true not only of rabbits, but in one instance of a human. This being true, the virus in the blood must occur in very small numbers, for ordinary methods, such as corneal and skin inoculations with blood, are always negative. In view of these facts we felt that it would be of interest to use as an inoculating material the plasma of an infected rabbit, for should our cultures prove positive on corneal or skin inoculation with this plasma as the sole source of the virus, it would show that definite growth of the virus had taken place presumably in the absence of primarily infected cells. Therefore, cultures were set up using the plasma of a rabbit inoculated (cornea, skin and testis) with vaccine virus five days previously, and pieces of normal rabbit testis. The cultures were incubated and transferred in the usual way. The third generation, inoculated on the cornea of a rabbit, was strongly positive macroscopically and showed many typical Guarnieri bodies microscopically.

### *Duration of Virus Growth in Artificial Medium*

In the former piece of work, vaccine virus was successfully cultivated for a total period of fifty-four days, at which time the work was discontinued. We decided to attempt to discover how long we could cultivate the virus. A culture, VC 6 A, was started June 25, 1924, using normal plasma and pieces of normal rabbit testis that had been soaked for five minutes in a heavy emulsion in Ringer's solution of a seventy-one-day-old, glycerinated, virus-infected rabbit

testis. This culture was incubated, transferred, and tested in the usual way (Table I).

TABLE I

*Growth Record of Vaccine Virus Culture, VC 6 A*

| Date | | Generation | Result of testing |
|---|---|---|---|
| 6/24/24 | Started | I | |
| 7/1 | Transfer | II | |
| 7/7 | " | III | |
| 7/11 | " | IV | Cornea + |
| 7/15 | " | V | |
| 7/21 | " | VI | |
| 7/25 | " | VII | |
| 7/30 | " | VIII | |
| 8/5 | " | IX | Cornea +; Skin + |
| 8/11 | " | X | |
| 8/15 | " | XI | |
| 8/20 | " | XII | |
| 8/27 | " | XIII | |
| 9/3 | " | XIV | |
| 9/10 | " | XV | |
| 9/15 | " | XVI | |
| 9/19 | " | XVII | |
| 9/24 | " | XVIII | Cornea +; Skin + |
| 9/29 | " | XIX | |
| 10/3 | " | XX | |
| 10/9 | " | XXI | |
| 10/15 | " | XXII | |
| 10/22 | " | XXIII | |
| 10/28 | " | XXIV | Cornea o; Skin o; Testis + Emulsion of this rabbit's testis inoculated on another rabbit's cornea, skin and testis; all were +. |
| 11/3 | " | XXV | |
| 11/7 | " | XXVI | |
| 11/13 | " | XXVII | |
| 11/19 | " | XXVIII | |
| 11/25 | " | XIX | |
| 12/1 | " | XXX | |
| 12/5 | " | XXXI | Cornea o; Skin o |
| 12/11 | " | XXXII | Cornea o; Skin o; Testis o Emulsion of this rabbit's testis inoculated on another rabbit's cornea, skin and testis; all were o. |
| 12/16 | " | XXXIII | |
| 12/22 | " | XXXIV | |
| 12/27 | " | XXXV | |
| 1/2/25 | " | XXXVI | Cornea o; Skin o; Testis o Tested 3/6/25 for immunity as described below. |

As will be seen from the above table, the fourth generation, inoculated on a rabbit's cornea, was positive macro- and microscopically; the ninth generation tested on the cornea was positive and a dilution equivalent to $\frac{1}{4600}$ of the culture injected intradermally was positive. The twenty-fourth generation was negative on the cornea and skin, but positive macroscopically in the testis. This testis was removed, an emulsion made and inoculated on another rabbit's cornea, skin and testis. All of the latter were positive macroscopically and the cornea and skin were positive microscopically. This result with the twenty-fourth generation may have been due to decreasing virulence of the virus, or to a diminished number of organisms, for the amount inoculated into the testis was several times that inoculated on the cornea and skin; however, the possibility of the development of a tissue affinity on the part of the virus due to continued cultivation in testicular tissue should be taken into consideration. The thirty-first generation, inoculated on the cornea and skin, was negative macroscopically. The thirty-second generation, inoculated on the cornea, skin and testis, was negative macroscopically in all; however, the testis was emulsified and inoculated on another rabbit's cornea, skin and testis. All these were negative macro- and microscopically. The thirty-sixth generation was negative on the cornea, skin and testis; this rabbit was tested for immunity on March 6, fifty-seven days after inoculation. This was done by inoculating on both corneas, skin and testis with an emulsion of glycerinated, vaccine-infected testis, and also on the skin with commercial vaccine virus. Both corneas and both testes were positive macro- and microscopically; the skin both with the commercial virus and the testis emulsion showed what was similar to an accelerated take in the human; that is, a marked take at the end of twenty-four hours, which was fading at seventy-two hours. Another rabbit, previously inoculated with positive results, and a normal rabbit were inoculated at the same time with both viruses; the revaccinated animal also showed an accelerated take while the reaction in the normal did not reach its height until the fourth day. Therefore it would appear that the rabbit inoculated with the thirty-sixth generation showed evidence of immunity as far as the skin was concerned. We carried out this experiment in this way as we felt that there was a possibility either that the virus might be present in too small amounts to give a positive take but in sufficient amounts to confer immunity, or that it

had lost its infecting power but had preserved its immunizing power. To sum up this cultivation experiment, the virus was demonstrated to be present in the fourth, ninth, eighteenth and twenty-fourth generations, covering a period of one hundred and thirty-two days of incubation; it did not give a positive take with the thirty-first, thirty-second and thirty-sixth generations; but the last apparently brought about some skin immunity on inoculation into a rabbit. The thirty-sixth generation represents a period of one hundred and ninety-eight days' incubation.

One experiment was undertaken to determine the amount of virus present in the ninth generation. The tissue of one of the cultures was weighed (5.9 mg.), ground with sand and 1.0 c.c. of Ringer's solution and centrifuged. Various dilutions of the supernatant fluid were made. One-tenth c.c. of each of these dilutions were injected intradermally into a rabbit. A dilution representing $\frac{1}{4000}$ of the culture (0.0013 mg. of culture) gave a positive skin test. Unfortunately this represented the maximum dilution and so the exact limit of positivity was undetermined.

### *Proof of Multiplication of Virus*

We have felt all along that, while experiments, such as the one just mentioned and the one where we started with infected plasma, proved that the virus actually multiplied in our cultures, we should endeavor to devise some method whereby the amount of virus present in any particular culture could be determined. Obviously corneal and skin inoculations with scarification are unsatisfactory, as the amount of inoculated material that actually comes in contact or remains in contact with the tissues long enough to infect, must vary tremendously; furthermore the depth and number of scarifications are other variable factors. Intradermal injection seemed to offer a method wherein the amount injected and site of injection could be most accurately controlled, and this method has proved most satisfactory.

The technique of titration is as follows: the whole culture (tissue and plasma) is ground up with 1.0 c.c. Ringer's solution in a sterile test-tube using a sterile glass rod and sterile sand; it is then centrifuged at moderate speed for five minutes, the clear or slightly opalescent supernatant fluid pipetted off and the desired dilutions of the latter made; these dilutions are injected intradermally in 0.1 c.c.

(rarely 0.2 c.c.) amounts using a tuberculin syringe and a fine (No. 26 gauge) needle.

This method of titration was carried out with culture VC 10 and the results are given in Table II. The original culture was started with pieces of normal rabbit testis and normal plasma to which had

TABLE II

*Growth and Titration of Vaccine Virus Culture, VC 10*

| Generation | Date | Split | Titration | | | Taking Skin Doses Contained per Culture | Virus Content Relative to Original Inoculum |
|---|---|---|---|---|---|---|---|
| | | | Date | Amt. Used | Result | | |
| I | 11–25–24 | | 11–25–24 | 1 culture* | 0.2 c.c undil. + 0.1 c.c. undil. − | 2½* | 1 |
| II | 12– 1–24 | | 12– 5–24 | 1 culture* | 0.2 c.c. undil. − | <2½* | <1 |
| III | 12– 5–24 | | | | | | |
| IV | 12–11–24 | | 12–16–24 | 2 cultures | 0.1 c.c. 1–50 + 0.1 c.c. 1–100 not done | 250 | 10 |
| V | 12–16–24 | Yes | | | | | |
| VI | 12–22–24 | | 12–27–24 | 2 cultures | 0.1 c.c. 1–200 + 0.1 c.c. 1–400 − | 1,000 | 800 |
| VII | 12–27–24 | | | | | | |
| VIII | 1– 2–25 | Yes | 1– 8–25 | 4 × ½ culture | 0.1 c.c. 1–400 + 0.1 c.c. 1–800 − | 2,000 | 1,600 |
| IX | 1– 8–25 | | | | | | |
| X | 1–14–25 | Yes | | | | | |
| XI | 1–20–25 | | 1–26–25 | 2 cultures | 0.1 c.c. 1–3200 + 0.1 c.c. 1–6400 not done | 16,000 | 51,000 |

* These two cultures contained originally 2 drops of virus plasma mixture instead of 1 drop. In each instance the "taking skin dose" is calculated on the assumption that only 1 drop was used.

been added such an amount of glycerinated vaccine-infected testis that the plasma virus mixture represented a dilution of about 1: 10 of the undiluted testicular suspension. Each piece of testis was covered with one drop of the plasma virus mixture, except for two pieces which received two drops apiece. One of the latter cultures, as soon as clotting had taken place, was removed, ground in 1.0 c.c. of Ringer's solution and titrated on a rabbit; 0.2 c.c. of the undiluted suspension gave rise to one vesicle. The other inoculations were all negative. Hence, this control culture contained a sufficient amount of virus to cause five positive intradermal reactions and all of the

other cultures, with the exception of the second one, which received 2 drops of plasma virus mixture, contained theoretically on the basis of this titration sufficient virus to cause two and a half positive intradermal reactions.

The cultures were then incubated and transferred as shown in Table II. When transferring, the tissue was freed as much as possible from its surrounding plasma. Obviously this method resulted in the loss at each transfer of a certain amount of virus; this loss has not been taken into account when figuring the relative strength of the original culture and the subsequent generations. If the whole culture could have been transferred each time, instead of only the tissue, the virus content of each generation would have been greater than that found. By the term "split" is meant that, as the amount of tissue in a culture became too large for satisfactory growth, this tissue was divided in half as evenly as possible and each half carried on as an individual culture, with the result that each culture of the subsequent generation contained only one half as much virus as before the splitting occurred.

Inspection of the column in Table II labeled "Taking Skin Doses per Culture" reveals the fact that a continuous increase in the virus content per culture took place from the third generation on. The reduction in virus content in the second generation was probably due to the fact that a considerable part of the virus had died, for it is known that incubation of the virus in plasma diminishes its strength rather rapidly. Between the second and fourth generations the virus evidently began growing well, for the fourth generation contained ten times as much virus as the original. From this generation onward multiplication occurred until in the eleventh generation the virus content per culture was 51,000 times that of the original. This figure was calculated as follows:

$$16{,}000\frac{\left\{\begin{array}{l}\text{number of skin taking}\\ \text{doses in 11th generation}\end{array}\right\}}{2.5\left\{\begin{array}{l}\text{number of skin taking}\\ \text{doses in first culture}\end{array}\right\}} \times 8\left\{\begin{array}{l}\frac{1}{8}=\text{portion of first}\\ \text{culture in 11th gen-}\\ \text{eration due to}\\ \text{splitting}\end{array}\right\} = 51{,}000$$

The average weight of our cultures was 10 mgm. Therefore, in the eleventh generation, $\frac{1}{1600}$ mgm. of the culture contained one taking skin dose, or one gram of this culture would yield 1,600,000 taking skin doses, whereas one gram of the original contained only 250 such doses.

This culture after the eleventh generation was unfortunately contaminated with moulds and had to be discarded.

### *Location of Virus Growth*

There has been throughout the work a question in our minds as to whether the virus grows within the cells, near the cells or at a distance from the cells in the plasma clot with which the tissue is covered. In an experiment reported in the preliminary note, it was shown that a normal piece of testis was infected by being placed in the same clot as an infected piece of testis although a space of 2 or 3 mm. of plasma intervened. In this experiment infection of the piece of normal testis might have taken place through (1) growth of the virus through the plasma, (2) migration of the virus through the plasma, (3) migration of infected cells through the plasma, or (4) transfer of the virus in the serum squeezed out of the clot. In order to throw light on the region of growth, a piece of virus tissue culture was placed in a plate, and covered with plasma, a projection of plasma about 1.0 cm. long being made at one side. After seven days' incubation the tissue and the plasma prolongation were removed separately and each was weighed, emulsified in Ringer's solution and similar dilutions made with regard to the weight of each piece. These various dilutions were then tested on a rabbit. The testis emulsion was positive with a dilution representing $\frac{1}{4600}$ of the whole, whereas the plasma emulsion was negative throughout. This experiment suggests that the growth of the virus takes place in intimate connection with the tissue. Histological investigations of our cultures have failed to throw any light on location of the virus or its morphology, so at present we cannot say whether growth is intra- or extracellular.

### *Unsuccessful Attempts to Cultivate Virus*

It seems worth while to record some of our unsuccessful attempts to cultivate vaccine virus in media other than the one used in the above experiments.

1. Pieces of culture testis from VC 6 A, sixth generation, were cultivated in serum agar. These were transferred every sixth day to fresh medium of the same composition. The sixth transfer was tested on a rabbit's cornea and proved negative macro- and microscopically.

2. A piece of culture testis from VC 6 A, sixth generation, was placed on the side of a 100 × 31 mm. test tube, about 0.5 cm. from the bottom. To this was added normal diluted rabbit plasma, the tube was slanted and the plasma allowed to clot. The tube was then placed upright and 2.0 c.c. of Ringer's solution added. After six days' incubation two drops of the Ringer's solution were transferred to another tube of the same composition, except that normal rabbit testis was used instead of culture testis. Two drops were also added as controls to a tube of Ringer's solution and plasma, and to another of Ringer's solution and normal testis. After five days' incubation the Ringer's solution in the original tubes and the Ringer's solution in the various tubes of the last transfer were tested on rabbits' corneas and all found negative macro- and microscopically.

This experiment was repeated incubating the tubes anaerobically, as well as aerobically, using as virus VC 6 A, ninth generation. After three transfers the fluids in both the anaerobic and aerobic cultures were tested intradermally in a rabbit and were negative.

3. As Carrel and other investigators have shown that chick embryonic tissue can be cultivated in artificial media for long periods of time, we thought this tissue might prove useful for our purposes. In a Carrel[4] "D" plate were placed several pieces of a fourteen day old chick embryo; these were covered with a mixture composed of 1.0 c.c. normal rabbit plasma, 0.15 c.c. chick embryonic juice, 0.15 c.c. emulsion of glycerinated vaccine-infected testis and 0.5 c.c. Ringer's solution. After clotting, 1.0 c.c of a Ringer's solution dilution (1:15) of chick embryonic juice was added. A second plate similar to the first except that no virus was added was set up as a control on tissue growth. The supernatant fluid was removed every three days, the cultures washed with Ringer's solution as recommended by Carrel and fresh chick embryonic juice diluted with Ringer's solution added. Tissue growth in both plates was good. At the end of 19 days' incubation (6 washings) the supernatant fluid, plasma and tissue of the virus culture were each tested separately by intradermal injections on a rabbit and all were found negative.

4. As embryonic juice is known to be a growth stimulant, it seemed advisable to try its effect on the virus using normal rabbit tissue rather than embryonic chick tissue. The cultures were set up in 100 × 13 mm. test-tubes, a piece of normal rabbit testis being

placed in the bottom. To each tube was added a mixture containing 0.25 c.c. 1:5 Ringer's solution dilution of virus culture VC 10, tenth generation, ground up in 1.0 c.c. Ringer's solution, 0.25 c.c. chick embryonic juice and 2.5 c.c. Ringer's solution. One tube was incubated aerobically, the other anaerobically. At the end of six days 0.25 c.c. of the fluid from the bottom of each tube was transferred to a tube containing similar amounts of normal testis, chick embryonic juice and Ringer's solution, and the tubes were incubated aerobically and anaerobically as before. At the same time some of the fluid from each of the original tubes was tested intradermally on a rabbit and found to be negative. The cultures were transferred after three days and again at the end of six days; at this time the fluid in each tube was again tested intradermally on a rabbit with negative results. After two more days' incubation, the piece of testis in each tube was emulsified in the fluid part of the culture and these emulsions were injected intradermally into a rabbit, and found to be negative.

## Summary

1. Vaccine virus can be cultivated in tissue cultures composed of normal tissue; the presence of previously infected, living cells is not necessary.

2. Vaccine virus was successfully cultivated in an artificial medium for one hundred thirty-two days. After one hundred ninety-eight days, the virus was not demonstrable by the usual methods, but apparently gave rise to a certain degree of skin immunity as tested by revaccination.

3. A method has been devised by means of which the virus content of any culture can be determined with a fair degree of accuracy. Using this method, the eleventh generation of one culture was shown to contain 51,000 times as much virus as the material from which it was started.

4. Our results suggest that the virus grows only in close proximity to the cells. Whether this growth takes place intra- or extracellularly cannot be stated at this time.

5. Various other methods for the cultivation of vaccine virus were tried without success.

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# STUDIES ON FILTERABLE VIRUSES *

## II. CULTIVATION OF HERPES VIRUS

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The results obtained in our attempts to cultivate vaccine virus, as reported in the preceding paper, seemed to justify the expectation that this method might be applied with hopes of success to the cultivation of others of the so-called filterable viruses. For this purpose we have used herpes virus and a note on the cultivation of this virus is given below.

The strain, "M," used was very kindly furnished us by Dr. E. W. Goodpasture. The material for the first culture, H 18, was obtained from a rabbit inoculated intracerebrally with glycerinated herpes-infected rabbit brain. The animal was killed in the convulsive stage and the brain removed aseptically. To each small piece of brain tissue was added a piece of normal rabbit testis, for we felt that the growth of the brain tissue might be insufficient to encourage growth of the virus. The plasma was obtained from the herpes-infected rabbit. At each transfer fresh normal rabbit testis and plasma were added, as with our vaccine virus cultures. The cultures were "split" whenever too much tissue for good tissue growth accumulated.

As a source of virus for our second and third cultures, H 19 and HC 3, we used rabbits' testes that had been inoculated forty-eight hours previously with an emulsion of the brain of a herpes-infected rabbit. In the fourth culture, HC 4, we used the testis of a rabbit that had been injected intratesticularly forty-eight hours previously with an emulsion of HC 3, tenth generation.

The complete records of these cultures with the results of testing are given in Table I. In each instance the macroscopic results were controlled microscopically. A positive diagnosis was not made unless we found typical, intranuclear inclusions in epithelial cells of the cornea or in the interstitial cells of Leydig of the testis, a location for the latter first described by Goodpasture and Teague.[1]

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As a control, to rule out the question of survival, one of the original plates was kept in the incubator without transferring. After fourteen days rabbit inoculation showed that the virus was dead, or at least present in insufficient amounts to give positive tests. This particular experiment was repeated with the same result. Therefore, we can safely say that incubation of the virus at 37.5° C. in plasma for two weeks either kills the virus or diminishes it to such an extent that positive takes cannot be obtained. From Table I it will be seen that in all the experiments the minimal incubation period at the time of first testing the cultures equaled or exceeded fourteen days.

We were able to cultivate one culture, HC 3, for a minimal total period of fifty-four days. No tests were made between the tenth and fifteenth transfers so that we do not know at what point the virus died. It seems possible that the virus may have survived for a longer period than that demonstrated.

TABLE I

*Culture and Inoculation Records of Herpes Virus Cultures*

| Culture | Date | Generation | | Result of Testing |
|---|---|---|---|---|
| H 18 ..... | 7/5/24 | Started | I | |
| | 7/11 | Transfer | II | |
| | 7/15 | " | III | |
| | 7/21 | " | IV | |
| | 7/25 | " | V | 7/30 Cornea + |
| | 7/30 | " | VI | |
| | 8/5 | " | VII | |
| | 8/11 | " | VIII | |
| | 8/15 | " | IX | |
| | 8/20 | " | X | |
| | 8/27 | " | XI | 9/3 Brain o; Cornea o |
| | 9/3 | " | XII | |
| | 9/10 | " | XIII | |
| | 9/15 | " | XIV | |
| H 19 ..... | 7/10/24 | Started | I | |
| | 7/15 | Transfer | II | |
| | 7/21 | " | III | |
| | 7/25 | " | IV | 7/30 Cornea + |
| | 7/30 | " | V | |
| | 8/4 | " | VI | |
| | 8/9 | " | VII | |
| | 8/11 | " | VIII | |
| | 8/15 | " | IX | 8/20 Cornea o |
| | 8/20 | " | X | |

TABLE I (*continued*)

| Culture | Date | | Generation | Result of Testing |
|---|---|---|---|---|
| HC 3 .... | 9/10/24 | Started | I | |
| | 9/15 | Transfer | II | |
| | 9/19 | " | III | 9/24 Cornea +; Testis + |
| | 9/24 | " | IV | |
| | 9/29 | " | V | |
| | 10/3 | " | VI | 10/9 Cornea + |
| | 10/9 | " | VII | |
| | 10/15 | " | VIII | |
| | 10/22 | " | IX | |
| | 10/28 | " | X | 11/3 Cornea +; Testis + |
| | 11/3 | " | XI | |
| | 11/7 | " | XII | |
| | 11/13 | " | XIII | |
| | 11/19 | " | XIV | |
| | 11/25 | " | XV | 12/1 Cornea o; Testis o |
| | 12/1 | " | XVI | |
| HC 4 .... | 11/5 | Started | I | |
| | 11/7 | Transfer | II | |
| | 11/13 | " | III | 11/19 Cornea o; Testis o |
| | 11/19 | " | IV | |
| | 11/25 | " | V | 12/1 Cornea + |
| | 12/1 | " | VI | |
| | 12/5 | " | VII | |
| | 12/11 | " | VIII | |
| | 12/16 | " | IX | Cornea o |

As yet no definite proof of multiplication can be presented, for no method of titration has been evolved that does not necessitate the use of an excessive number of animals.

In one culture, HC 4, while the third generation was negative, the fifth generation was positive, which suggests that multiplication must have taken place.

The reason for the death or loss of virulence of the virus is not yet clear. One fact has been noted which may have some bearing on this question: namely, after several transfers the fibroblasts in the fresh normal testis added each time grow very poorly or not at all. It may be that the virus in growing kills or injures the fibroblasts, and the poor growth of these in turn results in the eventual death of the virus. The fact that herpetic intranuclear inclusions occur in fibroblasts, as well as in epithelial cells, suggests that the virus has some affinity for connective tissue cells. With HC 3 one culture showed no fibroblastic growth, whereas another of the same generation showed very good growth. These two cultures preserved

these same characteristics for six weeks during which time they were transferred seven times. In this period each culture was "split" twice and the daughter cultures of each preserved the same characteristics. No virus could be demonstrated in either by the usual methods. It seems probable that either we had an attenuated form of the virus to account for this inhibition or the tissues themselves had developed some substance inhibitory to the growth of fibroblasts, possibly akin to the bacteriophage.

## REFERENCES

1. Goodpasture, E. W. and Teague, O. Jour. Med. Res., 1923, xliv, 121.